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HEREGULIN VARIANTS

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to heregulin variants, nucleic acid molecules encoding such variants, and related vectors, host cells, pharmaceutical compositions, and methods.

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In particular, the invention relates to amino acid substitution variants of human heregulin- β 1 having an enhanced affinity for the ErbB-3 and ErbB-4 receptors.

5 Description of the Related Art

Transduction of signals that regulate cell growth and differentiation is regulated in part by phosphorylation of various cellular proteins. Protein tyrosine kinases are enzymes that catalyze this process. Receptor protein tyrosine kinases are believed to direct cellular growth via ligand-stimulated tyrosine phosphorylation of intracellular proteins. Growth factor receptor protein tyrosine kinases of the class I subfamily include the 170 kilodalton (kDa) epidermal growth factor receptor (EGFR) encoded by the *erbB1* gene. *erbB1* has been causally implicated in human malignancy. In particular, increased expression of this gene has been observed in more aggressive carcinomas of the breast, bladder, lung, and stomach.

The second member of the class I subfamily, p185^{neu} (also called the ErbB-2 receptor or p185^{HER2}), was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The *neu* (*erbB2* or HER2) gene encodes a 185 kDa receptor protein tyrosine kinase.

Amplification and/or overexpression of the human *erbB2* gene correlates with a poor prognosis in breast and ovarian cancers. Slamon et al., *Science* 235:177-82 (1987); Slamon et al., *Science* 244:707-12 (1989). Overexpression of *erbB2* has been correlated with other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon and bladder. Accordingly, in U.S. Patent No. 4,968,603, Slamon et al. describe and claim various diagnostic assays for determining *erbB2* gene amplification or expression in tumor cells. Slamon et al. discovered

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that the presence of multiple copies of the *erbB2* oncogene in tumor cells indicates that the disease is more likely to spread beyond the primary tumor site, and that the disease may therefore require more aggressive treatment than might otherwise be indicated by other diagnostic factors. Slamon *et al.* conclude that the *erbB2* gene amplification test, together with the determination of lymph node status, provides greatly improved prognostic utility.

A further related gene, called *erbB3* (or *HER3*), which encodes the ErbB-3 receptor (p180^{HER3}) has also been described. See U.S. Patent No. 5,183,884; Kraus *et al.*, *PNAS USA* 86:9193-97 (1989); EP Patent Application No. 444,961A1; Kraus *et al.*, *PNAS USA* 90:2900-04 (1993). Kraus *et al.* (1989) discovered that markedly elevated levels of *erbB3* mRNA were present in certain human mammary tumor cell lines indicating that *erbB3*, like *erbB1* and *erbB2*, may play a role in human malignancies. Also, Kraus *et al.* (1993) showed that EGF-dependent activation of the ErbB-3 catalytic domain of a chimeric EGFR/ErbB-3 receptor resulted in a proliferative response in transfected NIH-3T3 cells. Furthermore, these researchers demonstrated that some human mammary tumor cell lines display a significant elevation of steady-state ErbB-3 receptor tyrosine phosphorylation, further implicating this receptor in human malignancies. The role of *erbB3* in cancer has been explored by others, and this gene has been found to be overexpressed in breast (Lemoine *et al.*, *Br. J. Cancer* 66:1116-21 [1992]), gastrointestinal (Poller *et al.*, *J. Pathol.* 168:275-80 [1992]; Rajkumar *et al.*, *J. Pathol.* 170:271-78 [1993]; Sanidas *et al.*, *Int. J. Cancer* 54:935-40 [1993]), and pancreatic cancers (Lemoine *et al.*, *J. Pathol.* 168:269-73 [1992], and Friess *et al.*, *Clinical Cancer Research* 1:1413-20 [1995]).

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The class I subfamily of growth factor receptor protein tyrosine kinases has been further extended to include the ErbB-4 (HER4) receptor, which is the product of the *erbB4* (HER4) gene. See EP Patent Application No. 599,274; Plowman et al., *PNAS USA* 90:1746-50 (1993); and Plowman et al., *Nature* 366:473-75 (1993). Plowman et al. found that increased *erbB4* expression closely correlated with certain carcinomas of epithelial origin, including breast adenocarcinomas. Diagnostic methods for detection of human neoplastic conditions (especially breast cancers) that evaluate *erbB4* expression are described in EP Patent Application No. 599,274.

The quest for the activator of the *erbB2* oncogene has lead to the discovery of a family of heregulin polypeptides. In humans, the heregulin polypeptides characterized thus far are derived from alternate splicing of a single gene which was mapped to the short arm of chromosome 8 by Lee and Wood, *Genomics* 16:790-91 (1993).

Holmes et al. isolated and cloned a family of polypeptide activators for the ErbB-2 receptor which they called heregulin- α (HRG- α), heregulin- β 1 (HRG- β 1), heregulin- β 2 (HRG- β 2), and heregulin- β 3 (HRG- β 3). See Holmes et al., *Science* 256:1205-10 (1992); WO 92/20798; and U.S. Patent No. 5,367,060. These researchers demonstrated the ability of the purified heregulin polypeptides to activate tyrosine phosphorylation of the ErbB-2 receptor in MCF7 breast tumor cells. Furthermore, the mitogenic activity of the heregulin polypeptides on SK-BR-3 cells (which express high levels of the ErbB-2 receptor) was also demonstrated.

Heregulins are large multi-domain proteins that are typically expressed as "pro-heregulins." Pro-heregulins have been shown to undergo proteolytic processing to a mature soluble form (usually of about

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44-45 kDa). Processing has been shown to occur intracellularly or at the cell surface. Domains in the soluble form include (in order from the N- to the C-terminus) an immunoglobulin homology (Ig-like) domain, a spacer region rich in glycosylation sites, and a domain similar to a domain found in EGF that is sufficient for ErbB receptor binding and activation. See Barbacci, et al., *J. Biol. Chem.* 270:9585-89 (1995).

10 The heregulin EGF-like domains are characterized by substantial structural similarities to (Jacobsen et al., *Biochemistry* 35:3402-17 [1996]), and limited sequence homology with, EGF residues 1-48 (Holmes, et al., *supra*). Functional similarities between the heregulin EGF-like domains and EGF have been established by data showing that blocks of EGF sequence substituted into heregulin- β 1 do not impair binding to cells co-expressing ErbB-3 and ErbB-2. Barbacci et al., *supra*.

20 While heregulins are substantially identical in the first 213 amino acid residues, they are classified into two major types, α and β , based on two EGF-like domains that differ in their C-terminal portions. For example, the heregulin- α EGF-like domain differs from that of the β 1-isoform by nine substitutions near the C-terminus. The β -isoform has been reported to bind ErbB receptors with approximately eight to 10-fold higher affinity than the α -isoform. Wen et al., *Mol. Cell. Biol.* 14:1909-19 (1994).

30 The solution structure of the heregulin- α EGF domain has recently been determined at high resolution by NMR. Jacobsen et al., *supra*; Nagata et al., *EMBO J.* 13, 3517-3523 (1994). The salient features of this domain include (1) an N-terminal subdomain containing a central three-stranded β -sheet with an intermittent helix and (2) a smaller C-terminal subdomain that contains a short stretch of β -sheet. The EGF domain is

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5 stabilized by three disulfide bonds, two in the N-terminal subdomain and one in the C-terminal subdomain. The pairing of the six corresponding cysteine residues is conserved in EGF-like domains from all heregulins and from EGF.

10 The 44 kDa neu differentiation factor (NDF), which is the rat equivalent of human HRG, was first described by Peles et al., *Cell*, 69:205-16 (1992), and Wen et al., *Cell*, 69:559-72 (1992). Like the human heregulin polypeptides, NDF has an Ig-like domain followed by an EGF-like domain and lacks a N-terminal signal peptide. Subsequently, Wen et al. carried out "exhaustive cloning" to extend the family of NDFs. Wen et al., *Mol. Cell. Biol.*, 14:1909-19 (1994). This work
15 revealed six distinct fibroblastic pro-NDFs. Adopting the nomenclature of Holmes et al., the NDFs were classified as either α or β polypeptides based on the sequences of the EGF-like domains. Isoforms 1 to 4 are characterized on the basis of a variable region between the EGF-like domain and transmembrane domain. Also,
20 isoforms a, b and c are defined based on variable-length cytoplasmic domains. These researchers conclude that different NDF isoforms are generated by alternative splicing and perform distinct tissue-specific functions. See also EP 505 148; WO 93/22424; and WO 94/28133 (discussing NDF).

Falls et al., *Cell* 72:801-815 (1993) describe another member of the heregulin family which they call "acetylcholine receptor inducing activity (ARIA)
30 polypeptide." The chicken-derived ARIA polypeptide stimulates synthesis of muscle acetylcholine receptors. See WO-94/08007. ARIA is a β -type heregulin and lacks the entire spacer region between the Ig-like domain and EGF-like domain of HRG- α and HRG β 1- β 3.

35 Marchionni et al., *Nature* 362:312-318 (1993) identified several bovine-derived proteins that they call "glial growth factors (GGFs)." These GGFs share

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as well as phosphorylation of ErbB-2 receptor in cells that express both receptors. Carraway et al., *J. Biol. Chem.* 269:14303-06 (1994). Sliwkowski et al. found that cells transfected with erbB3 alone show low
5 affinities for heregulin, whereas cells transfected with both erbB2 and erbB3 show higher affinities. Sliwkowski et al., *J. Biol. Chem.* 269:14661-65 (1994).

Plowman and his colleagues have similarly studied ErbB-4/ErbB-2 receptor activation. They expressed the
10 ErbB2 receptor alone, the ErbB4 receptor alone, or the two receptors together in human T lymphocytes and demonstrated that heregulin is capable of stimulating tyrosine phosphorylation of ErbB-4, but could only stimulate ErbB-2 phosphorylation in cells expressing
15 both receptors. Plowman et al., *Nature* 336:473-75 (1993).

These observations are consistent with the "receptor cross-talking" concept described previously by Kokai et al., *Cell* 58:287-92 (1989), Stern et al.,
20 *EMBO J.* 7:995-1001 (1988), and King et al., 4:13-18 (1989). These researchers found that binding of EGF to the EGFR resulted in activation of the EGFR kinase domain and cross-phosphorylation of the ErbB-2 receptor. This is believed to be a result of ligand-
25 induced receptor heterodimerization and the concomitant cross-phosphorylation of the receptors within the heterodimer. Wada et al., *Cell* 61:1339-47 (1990).

Thus, the ErbB receptors are believed to be activated by ligand-induced receptor dimerization.
30 Specifically, heregulins can bind separately to ErbB-3 and ErbB-4 receptors, but not to the ErbB-2 receptor. However, ErbB-2 is required for signalling, and heterodimers containing ErbB-2 in combination with ErbB-3 or ErbB-4 bind heregulins with higher affinity
35 than homodimers containing ErbB-3 or ErbB-4. Plowman et al., *Nature* 366:473-75 (1993); Sliwkowski et al., *J. Biol. Chem.* 269:14661-65 (1994).

The biological activities of heregulins have been investigated by several groups. For example, Holmes *et al.* (*supra*) found that heregulin exerts a mitogenic effect on mammary cell lines (such as SK-BR-3 and MCF-7). Lewis *et al.* reported that heregulin- β 1 stimulated proliferation and enhanced colony formation in soft agar in a number of human breast and ovarian tumor cell lines. Lewis *et al.*, *Cancer Research* 56:1457-65 (1996). These researchers also showed that ErbB-2 is a critical mediator of heregulin responsiveness.

Pinkas-Kramarski *et al.* found that NDF (rat heregulin) is expressed in neurons and glial cells in embryonic and adult rat brain and primary cultures of rat brain cells, and suggested that NDF may act as a survival and maturation factor for astrocytes. Pinkas-Kramarski *et al.*, *PNAS USA* 91:9387-91 (1994). Danilenko *et al.* reported that the interaction of NDF and the ErbB-2 receptor is important in directing epidermal migration and differentiation during wound repair. Danilenko *et al.*, Abstract 3101, *FASEB* 8(4-5):A535 (1994).

Meyer and Birchmeier analyzed expression of mouse heregulin during embryogenesis and in the perinatal animal using in situ hybridization and RNase protection experiments. Meyer and Birchmeier, *PNAS USA* 91:1064-68 (1994). These authors conclude, based on expression of this molecule, that heregulin plays a role in vivo as a mesenchymal and neuronal factor. Their findings also indicated that heregulin functions in the development of epithelia.

Falls *et al.* (*supra*) found that chicken ARIA plays a role in myotube differentiation, namely affecting the synthesis and concentration of neurotransmitter receptors in the postsynaptic muscle cells of motor neurons. Corfas and Fischbach demonstrated that ARIA

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also increases the number of sodium channels in chick muscle. Corfas and Fischbach, *J. Neuroscience* 13:2118-25 (1993).

Bovine GGFs have been reported to be mitogenic for Schwann cells. See, e.g., Brockes *et al.*, *J. Biol. Chem.* 255:8374-77 (1980); Lemke and Brockes, *J. Neurosci.* 4:75-83 (1984); Brockes *et al.*, *J. Neuroscience* 4:75-83 (1984); Brockes *et al.*, *Ann. Neurol.* 20:317-22 (1986); Brockes, *Methods in Enzym.* 147:217-225 (1987); Marchionni *et al.*, *supra*. Schwann cells provide myelin sheathing around the axons of myelinated neurons and thus play an important role in the development, function and regeneration of peripheral nerves. The implications of this role from a therapeutic standpoint have been addressed by Levi *et al.*, *J. Neuroscience* 14:1309-19 (1994). Levi *et al.* discussed the potential for construction of a cellular prosthesis including Schwann cells that could be transplanted into areas of damaged spinal cord. Methods for culturing Schwann cells *ex vivo* have been described. See WO 94/00140; Li *et al.*, *J. Neuroscience* 16:2012-19 (1996).

GGFII has been shown to be mitogenic for subconfluent quiescent human myoblasts, and differentiation of clonal human myoblasts in the continuous presence of GGFII results in greater numbers of myotubes after six days of differentiation. Sklar *et al.*, *J. Cell Biochem.*, Abst. W462, 18D, 540 (1994); see also WO 94/26298.

The relationship between the structure and function of new proteins can be investigated using any of a variety of available mutational analysis techniques. Examples of such techniques include alanine scanning mutagenesis and phagemid display. Alanine scanning can be used to identify active residues (i.e., residues that have a significant effect on protein function) in a protein or protein domain.

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For example, Cunningham and Wells used alanine scanning to identify residues in human growth hormone that were important for binding its receptor. Cunningham and Wells, *Science* 244:1081-85 (1989). In alanine scanning, a gene encoding the protein or domain to be scanned is inserted into an expression vector, and mutagenesis is carried out to generate a series of vectors that encode proteins or domains in which sequential residues are converted to alanine. The encoded proteins or domain are expressed from these vectors, and the activities of the alanine-substituted variants are then tested to identify those with altered activity. An alteration in activity indicates that the residue at the alanine-substituted position is an active residue.

Phagemid display was developed to allow the screening of a large number of variant polypeptides for a particular binding activity. Smith and Parmley demonstrated that foreign peptides can be "displayed" efficiently on the surface of filamentous phage by inserting short gene fragments into gene III of the fd phage. Smith, *Science* 228:1315-17 (1985); Parmley and Smith, *Gene* 73:305-18 (1985). The gene III coat protein is present in about five copies at one end of the phage particle. The modified phage were termed "fusion phage" because they displayed the foreign peptides fused to the gene III coat protein. As each fusion phage particle displayed approximately five copies of the fusion protein, this mode of phage display was termed "polyvalent display."

Scott et al. and Cwirla et al. showed that fusion phage libraries could be screened by sequential affinity selections known as "panning." Scott et al., *Science* 249:386-90 (1990); Cwirla et al., *PNAS USA* 87:6378-82 (1990). However, early efforts to select high affinity fusion phage failed, presumably due to the polyvalence of the phage particles. This problem

5 Bass et al., *Proteins* 8:309-14 (1990); Lowman et al.,
Biochem. 30:10832-38 (1991). Monovalent phage display
can be used to generate and screen a large number of
variant polypeptides to isolate those that bind with
high affinity to a target of interest.

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K185E, S, Q, or G; E186R; K187E or A; T188Q;
E195Q; F197Y; M198R or K; K200R; D201T or I;
P205T or Y; S206K, H, G, P, or R; R207Y;
Y208R or L; L209M or G; K211R; P213S, T, N,
5 or K; N214L, K, S, or E; F216M; N223H or W;
and M226I.

In a variation of this embodiment, the heregulin
variant includes sets of amino acid substitutions
selected from this group. Some heregulin variants of
10 the invention having sets of amino acid substitutions
exhibit at least a 50-fold increase in ErbB-3 receptor
affinity, which is also accompanied by an increase in
ErbB-4 receptor affinity.

The invention also includes a heregulin variant
15 that has a greater specificity for the ErbB-4 receptor,
relative to the ErbB-3 receptor, than the heregulin
from which the heregulin variant is derived. In one
embodiment, this heregulin variant has an amino acid
substitution at a selected residue corresponding to a
20 residue of 645-amino acid native human heregulin- β 1
selected from the group consisting of H178, L179, and
R207.

In another embodiment, a heregulin variant having
a greater specificity for the ErbB-4 receptor, relative
25 to the ErbB-3 receptor, has a deletion of amino acid
residues corresponding to residues S228 to K231 of
645-amino acid native human heregulin- β 1 and a
substitution of a single methionine for the deleted
residues.

30 In addition to including one or more of the
mutations disclosed herein, the heregulin variant can
have one or more other modifications, such as an amino
acid substitution, an insertion of at least one amino
acid, a deletion of at least one amino acid, or a
35 chemical modification. For example, the invention
provides a heregulin variant that is a fragment. In a
variation of this embodiment, the fragment includes

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residues corresponding to a portion of human heregulin- β 1 extending from about residue 175 to about residue 230 (i.e., the EGF-like domain). In a further variation of this embodiment, the fragment includes
 5 residues corresponding to a portion of human heregulin- β 1 extending from about residue 175 to about residue 245.

One aspect of the invention is a method for producing such a modified heregulin variant. The
 10 modification(s) is selected so that the modified heregulin variant retains the ability to an ErbB receptor.

In addition to a heregulin variant, the invention provides a related nucleic acid molecule, vector, and
 15 host cell. The invention also provides a method of producing a heregulin variant in which a host cell containing an expression vector capable of expressing the heregulin variant is cultured under conditions that allow expression of the heregulin variant, and the
 20 heregulin variant then recovered from the culture.

Other aspects of the invention relate to various uses of a heregulin variant. For example, the invention provides a method for activating an ErbB receptor in which the heregulin variant is contacted
 25 with a cell that expresses an ErbB receptor. The heregulin variant can be contacted with cells in culture, for example, to promote ex vivo survival, proliferation, or differentiation of cells, such as glial, Schwann, or muscle cells.

Alternatively, the heregulin variant can be
 30 combined with a pharmaceutically acceptable carrier and used to treat one of a wide range of cancers as well as diseases and disorders affecting the nervous system, musculature, and epithelia. Thus, the present
 35 invention provides a pharmaceutical composition and a treatment method.

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on phage. The EC_{50} was calculated as the concentration of soluble receptor fusion required to displace 50% of the total amount of phage bound to immobilized receptor fusion. ErbB-3 binding results are shown with black bars, and ErbB-4 binding results are shown with white bars.

Figure 3 shows the amino acids selected for binding to ErbB-3-Ig at each position in the heregulin- β 1 EGF-like domain (heregulin- β 1 residues 177-228) randomized in the phage display studies described in Example 3. The length of the bars indicates the frequency of occurrence of a particular amino acid at each position in the variants from phage display libraries A-E and G-I for which sequences were determined (i.e., a longer bar indicates a higher frequency). Twelve clones were sequenced from each library, although in library H, only one clone of the twelve represented a variant having mutations in the desired randomization window (see Example 3). "WT" indicates the wild-type amino acid sequence of the heregulin- β 1 EGF-like domain.

Figure 4 shows the amino acid substitutions in the EGF-like domains of combination variants described in Example 3. The amino acid sequence of the wild-type heregulin- β 1 EGF-like domain (HRG8), a variant of this domain containing a deletion of heregulin- β 1 residues 202-204 (HRG63), and the analogous domain in EGF are shown on top. The residue numbering for the portion of the heregulin- β 1 amino acid sequence shown is indicated above this sequence (numbered from the N-terminus of native human heregulin- β 1). The residue numbering for the portion of the EGF amino acid sequence shown is indicated below this sequence (numbered from the N-terminus of native human EGF). A "." indicates a residue that is identical to the wild-type residue at the particular position. A "-" indicates the absence of a residue.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides heregulin variants having one or more amino acid substitutions at selected residues. Included within the scope of the invention are variants of human heregulins, and, in particular, variants of human heregulin- β 1. A heregulin variant according to the invention can have a single amino acid substitution at a selected residue or combinations of such substitutions.

10 In addition to the amino acid substitutions specified herein, heregulin variants according to the invention can have further modifications, including, for example, deletions of amino acids. In one embodiment, a heregulin variant has N- and C-terminal deletions, leaving only amino acids corresponding to "the minimal EGF-like domain," which is sufficient for binding and activation of an ErbB receptor.

15 A heregulin variant of the invention is capable of binding an ErbB receptor, such as ErbB-3 or ErbB-4. In one embodiment, the variant has an enhanced affinity for an ErbB receptor compared to the affinity of the most homologous native heregulin. In addition to ErbB receptor binding, the heregulin variant can possess one or more other biological activities of a native heregulin.

25 The invention also provides nucleic acid molecules, vectors, and host cells related to the heregulin variants. A nucleic acid molecule of the invention encodes, or is complementary to a nucleic acid molecule encoding, a heregulin variant of the invention or a fragment thereof. The nucleic acid molecule can be double- or single-stranded DNA or RNA. A nucleic acid molecule of the invention can be inserted into an appropriate vector for propagation and/or expression of an encoded heregulin variant. Such vectors are introduced into suitable hosts, for

example, to allow recombinant production of a heregulin variant.

The heregulin variants of the invention are useful in a variety of therapeutic and non-therapeutic applications. In particular, heregulin variants can be used in treating cancer and various diseases and disorders of the nervous system, musculature, and epithelia. Accordingly, the invention encompasses a pharmaceutical composition including a heregulin variant and related treatment methods.

Heregulin variants can also be employed in a variety of non-therapeutic applications, such as cell culture methods and diagnostic methods. For example, heregulin variants can be used to promote the ex vivo survival, proliferation, or differentiation of cells, including glial and muscle cells. In an exemplary diagnostic application, heregulin variants are employed in the diagnosis of a cancer characterized by *erbB* (e.g., *erbB2*) overexpression. Accordingly, the invention also includes kits useful in practicing the above-described methods.

Definitions

As used herein, the following words or phrases have the definitions indicated below, unless otherwise indicated.

The terms "amino acid" and "residue" are used interchangeably herein.

The term "wild-type amino acid" or "wild-type residue" means the amino acid present at a given position(s) in a native polypeptide.

Amino acids are denoted herein by the standard three-letter or one-letter code.

Residues in two or more polypeptides are said to "correspond" if the residues occupy an analogous position in the polypeptide structures. As is well known in the art, analogous positions in two or more

polypeptides can be determined by aligning the polypeptide sequences based on amino acid sequence or structural similarities. Those skilled in the art understand that it may be necessary to introduce gaps
5 in either sequence to produce a satisfactory alignment. For example, residues in human EGF that correspond to residues in human heregulin- β 1 are shown in an alignment between the amino acid sequence of the heregulin- β 1 EGF-like domain (heregulin- β 1 residues
10 177-228) and the analogous EGF domain (EGF residues 1-48) in Figure 4.

Residues in two or more heregulins are said to "correspond" if the residues are aligned in the best sequence alignment. The "best sequence alignment"
15 between two polypeptides is defined as the alignment that produces the largest number of aligned identical residues. The best sequence alignment for a number of heregulin polypeptides is shown in Figure 1.

Residue positions in heregulin- β 1 are designated herein by the three-letter or one-letter code for the amino acid, followed by the position number, as
20 numbered from the N-terminus of native human pro-heregulin- β 1 (which is 645 amino acids in length). E.g., the serine at position 177 of heregulin- β 1 is
25 denoted "Ser177" or "S177."

Hereinafter, unless otherwise indicated, residue positions in a heregulin, heregulin variant, or related protein, such as EGF, are specified herein with reference to the amino acid numbering of native human
30 heregulin- β 1. For example, a heregulin- β 1 variant can have a N-terminal deletion of residues 1-176. The first amino acid in this variant is identified herein as "the residue corresponding to Ser177 of 645-amino acid human heregulin- β 1" because the first residue of
35 the heregulin variant and Ser177 of heregulin- β 1 are aligned in the best alignment between the two polypeptides.

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Example 3 discloses heregulin- β 1 variants containing residues corresponding to residues 177 to 228 of heregulin- β 1, which is termed "the minimal EGF-like domain." For these variants, residue numbers also are expressed, in parentheses, in terms of the position of the residue in the minimal EGF-like domain (hereinafter "heregulin- β 1 EGF" or "HRG- β 1 EGF"), i.e., residues 1-52. Residue positions numbered according to native human heregulin- β 1 can be converted to residue positions in the minimal EGF-like domain by subtracting 176 from the former position number. E.g., for heregulin- β 1 Ser177, subtracting 176 from 177 gives 1, and thus heregulin- β 1 EGF Ser1 identifies the same position as heregulin- β 1 Ser177. The same numbering system is used in Example 4.

Amino acid substitutions are indicated by listing the residue position followed by the code for the amino acid substituted into the heregulin polypeptide. Thus, a substitution of alanine at Ser177 of heregulin- β 1 is expressed as "heregulin- β 1 Ser177Ala," "Ser177Ala," or "S177A." In this example, serine is the "replaced amino acid," and alanine is the "replacement amino acid."

As used to describe two amino acid sequences, the term "homologous" indicates that the amino acid sequences have some degree of amino acid sequence identity.

Heregulin Variants

The present invention includes a heregulin variant. The term "heregulin variant" means a polypeptide variant of a native heregulin. A native heregulin is defined as a polypeptide having the full-length amino acid sequence of any of the family of naturally occurring heregulin polypeptides. This family encompasses pro-heregulins as well as the

soluble forms of these proteins. The invention is exemplified with variants of human heregulin- β 1. See Examples 1-3. However, the heregulin family encompasses any naturally occurring polypeptide having an EGF-like domain that has at least 70 percent sequence identity with the EGF-like domain of heregulin- β 1 when these domains are aligned in the best alignment. Thus, a native heregulin can be from any species and one of a number of naturally occurring isoforms or allelic forms. Exemplary heregulin polypeptides include *neu* differentiation factors, glial growth factors, sensory and motor neuron-derived factor, and acetylcholine receptor inducing activity polypeptide.

In one embodiment, the heregulin variant is a variant of a mammalian heregulin. In a variation of this embodiment, the heregulin variant is a variant of a human heregulin. Examples of human heregulins include heregulin- α (HRG- α), heregulin- β 1 (HRG- β 1), heregulin- β 2 (HRG- β 2), heregulin- β 3 (HRG- β 3), and heregulin- γ (HRG- γ).

A heregulin variant according to the invention has an amino acid sequence not found in nature in which a wild-type residue in a native heregulin is replaced with a different residue. This amino acid substitution is at one or more selected residues corresponding to a residue of native human heregulin- β 1. The selected residue(s) is chosen from the following group:

S177, H178, L179, V180, K181, E184, E186, K187, T188, V191, N192, G193, G194, E195, M198, V199, K200, D201, N204, P205, S206, R207, Y208, L209, K211, P213, N214, E215, T217, G218, D219, Q222, N223, Y224, S228, and F229.

In one embodiment, however, the amino acid substitution is not a replacement of the selected residue with an EGF residue corresponding to the selected residue.

5 In one embodiment, the selected residue is chosen
from the following group:

10 Substitution of the any of these residues in
heregulin- β 1 with alanine has at least a five-fold
effect on affinity for the ErbB-3 or ErbB-4 receptors,
as determined by phage ELISA. See Example 2.

H178, L179, K187, N192, G193, G194, E195,
R207, K211, T217, G218, Q222, and Y224.

Generally, if function is to be preserved at a position selected for substitution, the residue used to replace the selected residue is not substantially different in character from the wild-type residue, i.e., the amino acid substitution is a conservative substitution. Amino acids can be grouped according to character as shown in Table 1.

Table 1
Groups of Amino Acids
Having Similar Character

5	<u>Group</u>	<u>Character</u>	<u>Amino Acids</u>
	a	positively charged	Lys, Arg, His
	b	negatively charged	Asp, Glu
	c	amide	Asn, Gln
	d	aromatic	Phe, Tyr, Trp
10	e	hydrophobic	Pro, Gly, Ala, Val, Leu, Ile, Met
	f	uncharged hydrophilic	Ser, Thr

To preserve function, therefore, the residue used to replace the wild-type residue is usually selected from the same group or a related group. In addition, serine or alanine can be used to replace most other residues. Table 2 shows conservative substitutions for each amino acid, identifying related groups for each and indicating which amino acids can be replaced with serine or alanine. Table 2 also shows preferred amino acid substitutions.

Table 2
Conservative Amino Acid Substitutions

5	AA*	Replacement Amino Acid Selected From	Preferred Substitutions
10	Ala	e*: Pro, Gly, Ala, Val, Leu, Ile, Met f: Ser, Thr	Ser
	Arg	a: Lys, Arg, His Ser, Ala	Lys Ser, Ala
	Asn	a: Lys, Arg, His c: Asn, Gln	Gln
15	Asp	Ser, Ala b: Asp, Glu c: Asn, Gln	Ser, Ala Glu
	Cys	Ser, Ala e: Pro, Gly, Ala, Val, Leu, Ile, Met f: Ser, Thr	Ser, Ala Ala Ser
20	Gln	a: Lys, Arg, His c: Asn, Gln	Asn Ser, Ala
	Glu	Ser, Ala b: Asp, Glu c: Asn, Gln	Asp
25	Gly	Ser, Ala e: Pro, Gly, Ala, Val, Leu, Ile, Met f: Ser, Thr	Ser, Ala Pro, Ala
	His	a: Lys, Arg, His Ser, Ala	Arg Ser, Ala
30	Ile	e: Pro, Gly, Ala, Val, Leu, Met	Ala, Val, Leu
	Leu	e: Pro, Gly, Ala, Val, Ile, Met	Ala, Val, Ile
	Lys	a: Lys, Arg, His Ser, Ala	Arg Ser, Ala
35	Met	e: Pro, Gly, Ala, Val, Leu, Ile, Met	Ala, Val, Leu, Ile
	Phe	a: Lys, Arg, His d: Phe, Tyr, Trp Ala, Val, Leu, Ile	Tyr Ala, Val, Leu, Ile
40	Pro	a: Lys, Arg, His d: Phe, Tyr, Trp Gly, Ala	Phe Gly, Ala
	Ser	a: Lys, Arg, His f: Ser, Thr Ala	Thr Ala
45	Thr	a: Lys, Arg, His f: Ser, Thr Ala	Ser Ala
	Trp	d: Phe, Tyr, Trp Ala	Phe Ala
50	Tyr	d: Phe, Tyr, Trp Ala, Val, Leu, Ile	Phe, Ala, Val, Leu, Ile
	Val	e: Pro, Gly, Ala, Val, Leu, Ile, Met Ser, Ala	Leu, Ile, Ser, Ala

55 * Wild-type amino acid.

* Lower-case letters refer to the groups of amino acids in Table 1.

In one embodiment, the heregulin variant is a variant of human heregulin- β 1 and includes an amino acid substitution(s) chosen from the following group:

5 S177W; H178S, E, R, or A; V180Q, I, or E;
K181P or A; A183G, T, or D; E184V, W, K, R,
G, or N; K185E, S, Q, or G; E186R; K187E or
A; T188Q; E195Q; F197Y; M198R or K; K200R;
D201T or I; P205T or Y; S206K, H, G, P, or R;
R207Y; Y208R or L; L209M or G; K211R; P213S,
10 T, N, or K; N214L, K, S, or E; F216M; N223H
or W; and M226I.

Another aspect of the invention is a variant of human heregulin- β 1 including a set of amino acid substitutions, such as any of the heregulin variants
15 described in Example 3. The set of amino acid substitutions is chosen from the group indicated below (the variant number from Example 3 is shown in the left-hand column, followed by the set of amino acid substitutions for that variant):

20 B5: A183G, E184W, K185D, E186R, K187E, T188G, M226I;
B10: A183D, E184K, K185S, E186R, K187E, T188G, M226I;
D1: F197Y, M198K, K200R, D201I, M226I;
25 E2: P205Y, S206G, R207Y, Y208L, L209M;
E3: P205Y, S206R, R207Y, Y208R, L209M, M226I;
E6: P205T, S206H, R207Y, Y208R, L209M;
E8: P205T, S206K, R207Y, Y208R, L209G;
I1: N223W, M226I;
30 I2: N223H, M226I;
HRG37: S177W, H178E, K181P, A183G, E184W, K185D, E186R, K187E, T188G, M226I;
HPG48: P205Y, S206G, R207Y, Y208L, L209M, M226I;
HRG53: A183G, K185E, E186R, K187E, T188G, F197Y, M198R, D201T;
35 HRG54: A183G, K185E, E186R, K187E, T188G, P205Y, S206G, R207Y, Y208L, L209M;

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HRG55: A183G, K185E, E186R, K187E, T188G, F197Y,
M198R, D201T, P205Y, S206G, R207Y, Y208L,
L209M;
HRG56: A183G, K185E, E186R, K187E, T188G, M226I;
5 HRG57: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M;
HRG58: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M, M226I;
HRG59: F197Y, M198R, D201T, M226I;
10 HRG60: A183G, K185E, E186R, K187E, T188G, F197Y,
M198R, D201T, M226I;
HRG61: A183G, K185E, E186R, K187E, T188G, P205Y,
S206G, R207Y, Y208L, L209M, M226I;
HRG62: A183G, K185E, E186R, K187E, T188G, F197Y,
15 M198R, D201T, P205Y, S206G, R207Y, Y208L,
L209M, M226I;
HRG71: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M, N223H, M226I; and
HRG73: A183G, K185E, E186R, K187E, T188G, F197Y,
20 M198R, D201T, P205Y, S206G, R207Y, Y208L,
L209M, N223H, M226I.

Each of these sets of amino acid substitutions produces
at least a five-fold increase in ErbB-3 receptor
affinity, as determined by phage ELISA. See Example 3.

25 In a variation of this embodiment, the set of
amino acid substitutions is chosen from the following
group:

B5: A183G, E184W, K185D, E186R, K187E, T188G,
M226I;
30 E2: P205Y, S206G, R207Y, Y208L, L209M;
I2: N223H, M226I;
HRG48: P205Y, S206G, R207Y, Y208L, L209M, M226I;
HRG53: A183G, K185E, E186R, K187E, T188G, F197Y,
M198R, D201T;
35 HRG56: A183G, K185E, E186R, K187E, T188G, M226I;
HRG57: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M;

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HRG58: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M, M226I;
HRG59: F197Y, M198R, D201T, M226I;
HRG60: A183G, K185E, E186R, K187E, T188G, F197Y,
5 M198R, D201T, M226I;
HRG61: A183G, K185E, E186R, K187E, T188G, P205Y,
S206G, R207Y, Y208L, L209M, M226I;
HRG62: A183G, K185E, E186R, K187E, T188G, F197Y,
M198R, D201T, P205Y, S206G, R207Y, Y208L,
10 L209M, M226I;
HRG71: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M, N223H, M226I; and
HRG73: A183G, K185E, E186R, K187E, T188G, F197Y,
M198R, D201T, P205Y, S206G, R207Y, Y208L,
15 L209M, N223H, M226I.

Each of these sets of amino acid substitutions produces
at least a 20-fold increase in ErbB-3 receptor
affinity, as determined by phage ELISA. See Example 3.

In another variation of this embodiment, the set
20 of amino acid substitutions is chosen from the
following group:

HRG58: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M, M226I;
HRG60: A183G, K185E, E186R, K187E, T188G, F197Y,
25 M198R, D201T, M226I;
HRG71: F197Y, M198R, D201T, P205Y, S206G, R207Y,
Y208L, L209M, N223H, M226I; and
HRG73: A183G, K185E, E186R, K187E, T188G, F197Y,
M198R, D201T, P205Y, S206G, R207Y, Y208L,
30 L209M, N223H, M226I.

Each of these sets of amino acid substitutions produces
at least a 50-fold increase in ErbB-3 receptor
affinity, as determined by phage ELISA. See Example 3.

The invention also includes a heregulin variant
35 that has a greater specificity for the ErbB-4 receptor,
relative to the ErbB-3 receptor, than the heregulin
from which the heregulin variant is derived. Such

enhanced ErbB-4 receptor specificity can be measured, for example, as a lower variant:wild-type EC_{50} ratio for binding to the ErbB-4 receptor than for binding to ErbB-3 receptor. Generally, a heregulin variant having
5 enhanced ErbB-4 receptor specificity has approximately wild-type affinity for the ErbB-4 receptor or better together with a significantly reduced (i.e., at least about 5-10-fold) affinity for the ErbB-3 receptor.

In one embodiment, a heregulin variant having
10 enhanced ErbB-4 receptor specificity has an amino acid substitution at a selected residue corresponding to a residue of 645-amino acid native human heregulin- β 1 selected from the group consisting of H178, L179, and R207. For example, the invention provides a human
15 heregulin- β 1 variant including the amino acid substitution H178E or R207P.

In another embodiment, a heregulin variant having enhanced ErbB-4 receptor specificity has a deletion of amino acid residues corresponding to residues S228 to
20 K231 of 645-amino acid native human heregulin- β 1 and a substitution of a single methionine for the deleted residues. In a variation of this embodiment, the heregulin variant also includes the amino acid substitution H178L.

25 In addition to the above amino acid substitutions, the heregulin variant can optionally have any of the following modifications, singly or in combination:
(1) one or more additional amino acid substitutions;
(2) one or more amino acids added to the N- or
30 C-terminus of, or inserted within, the amino acid sequence of the heregulin variant; (3) one or more amino acids deleted from the heregulin variant; and
(4) one or more covalent modifications of an amino acid in the heregulin variant.

35 Thus, the heregulin variant can be "substantially full-length," which, as used herein, means that the heregulin variant is at least 90% as long as the native

heregulin to which the variant is most homologous. Alternatively, the heregulin variant can be a "fragment" that is less than 90% as long as the most homologous native heregulin. Heregulin variants that are fragments are usually about 30 to about 100 amino acids, more usually about 40 to about 60 amino acids, even more usually about 45 to about 65 amino acids, and most usually about 50 amino acids in length.

For example, the heregulin variant can include amino acids corresponding to "the minimal EGF-like domain." The minimal EGF-like domain is a portion of a native heregulin that is sufficient for binding and activation of an ErbB receptor. In general, the minimal EGF-like domain is less than about 70 amino acids and usually less than about 60 amino acids in length. As used herein with reference to human heregulin- β 1, the minimal EGF-like domain extends from residues 177-228. Unless otherwise indicated, "HRG- β 1 EGF" refers to the minimal EGF-like domain.

Examples of suitable covalent modifications of a heregulin variant according to the invention include, but are not limited to, conjugation with a detectable label, "pegylation," and conjugation with a cytotoxic agent. A heregulin variant can be conjugated to any of a wide variety of available labels to produce a conjugate useful for detecting the presence of ErbB receptors in a sample. Suitable labels include a radioisotope, a fluorescent label, and an enzyme label. Exemplary radioisotope labels are ^{35}S , ^{14}C , ^{125}I , ^3H , and ^{131}I . Heregulin variants can be conjugated to radioisotopes as described generally in Current Protocols in Immunology Vols. 1 & 2 (Coligen et al. ed., Wiley Publishers).

Fluorescent labels suitable for conjugation to a heregulin variant include a rare earth chelate (a europium chelate), fluorescein, rhodamine, dansyl, Lissamine, phycoerythrin, and Texas Red, and

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derivatives thereof. Conjugates can be prepared as described, for example, in Current Protocols in Immunology *supra*.

Various enzyme-substrate systems are available, and U.S. Patent No. 4,275,149 provides a review of some of these. In general, enzymes useful in such systems catalyze a readily detectable chemical alteration of a substrate. For example, the enzyme can catalyze a color change, which can be measured spectrophotometrically, or a change in fluorescence or chemiluminescence, which can be detected using a fluorometer or chemiluminometer, respectively. Exemplary enzyme labels include a luciferase, malate dehydrogenase, urease, a peroxidase, alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, a saccharide oxidase, a heterocyclic oxidase, lactoperoxidase, microperoxidase, and the like. Heregulin variants can be conjugated to enzyme labels as described generally in O'Sullivan et al., *Methods in Enzym.* 73:47-166 (1981), and in Current Protocols in Immunology (*supra*). Suitable substrates for use with a given enzyme label are well known to those skilled in the art.

Another exemplary modification of a heregulin variant of the invention is pegylation, which refers to the conjugation of one or more polyethylene glycol (PEG) groups to the ϵ -amino group(s) of a polypeptide. Pegylation may be desired when the heregulin variant is intended for pharmaceutical use, as pegylation can increase in vivo half-life and/or reduce immunogenicity and potential toxicity of therapeutic proteins. See, e.g., Abuchowski et al., *J. Biol. Chem.* 252:3582-86 (1977).

Conjugation of a heregulin variant with a cytotoxic agent produces a targeted cytotoxic agent that specifically binds cells expressing appropriate ErbB receptors on their surface. The term "cytotoxic

agent" refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term includes, for example, a radioactive isotope (e.g., I, Y, Pr) and a chemotherapeutic agent.

5 A "chemotherapeutic agent" is defined herein as any chemical compound useful in the treatment of cancer. The term "cancer" refers to the physiological condition in mammals that is characterized by unregulated cell growth. Examples of cancer include
10 but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, pancreatic cancer, glial cell tumors such as
15 glioblastoma and neurofibromatosis, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, renal cancer, prostate cancer, vulval cancer,
20 thyroid cancer, hepatic carcinoma and various types of head and neck cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil (5-FU), Cytosine arabinoside (Ara-C), Cyclophosphamide, Thiopeta, Busulfan, Cytosine, Taxol, Methotrexate,
25 Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, VP-16, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, a Mitomycin, Nicotinamide, an
30 Esperamicin, Melphalan and any related nitrogen mustard, and an endocrine therapeutic (such as diethylstilbestrol [DES], Tamoxifen, a leutinizing hormone releasing hormone-antagonizing drug, a progestin, an anti-progestin, etc.).

35 In addition to conjugation to a chemical compound, any of the above-described heregulin variants can be modified by fusion to a heterologous polypeptide to

produce a "chimeric heregulin variant." (Chimeric heregulin variants are also referred to herein as "fusion proteins.") Typically, the heterologous polypeptide is fused at the N- or C terminus of the heregulin variant to preserve the biological activity (described further below) of the heregulin variant. However, the heterologous polypeptide can also be introduced into regions of the heregulin variant that are not critical for biological activity. Generally, chimeric heregulin variants are produced by recombinant techniques. Examples of chimeric heregulin variants include a heregulin variant fused to a "signal sequence," a "purification handle" and an immunoglobulin sequence.

A "signal sequence" is an amino acid sequence that directs the secretion of a polypeptide fused thereto from a cell expressing the chimeric protein. Thus, fusion of a heregulin variant to a signal sequence facilitates recombinant production of the heregulin variant because the chimeric heregulin variant is secreted into the host cell culture medium, from which the chimeric heregulin variant can be recovered with relative ease.

A suitable signal sequence can be obtained from any protein that has a signal sequence and is typically (but not always) fused to the N-terminus of the heregulin variant. DNA encoding prokaryotic signal sequences can be obtained, for example, from *lamB* or *ompF*, *MalE*, *PhoA*, and other genes. A convenient prokaryotic signal sequence for practicing the invention is the *E. coli* heat-stable enterotoxin II (STII) signal sequence.

A "purification handle" is a portion of a polypeptide that binds another polypeptide, termed a "binding partner." The fusion of a purification handle to a heregulin variant confers on the variant the ability to bind the binding partner, which facilitates

purification of the resultant chimeric heregulin variant. Generally, the purification handle is selected so that the binding partner does not substantially cross-react with other components present in the mixture from which the chimeric heregulin variant is to be purified. As used herein, the term "does not substantially cross-react" means that the affinity of the binding partner for the purification handle is at least about 20-fold, usually at least about 100-fold, more usually at least about 1000-fold, any affinity for any other components present in the mixture.

In one embodiment, the purification handle is an epitope recognized by an antibody, and the chimeric heregulin variant is therefore termed an "epitope-tagged heregulin variant." Suitable epitopes generally have at least five amino acids, usually between about 10 and about 50 amino acids, and more usually between about 10 and about 30 amino acids.

A chimeric molecule that includes a heregulin variant fused to an immunoglobulin sequence is termed "a heregulin variant immunoadhesin." In one embodiment, the immunoglobulin sequence is an immunoglobulin constant domain. The immunoglobulin sequence in a heregulin variant immunoadhesin can be obtained from IgG₁, IgG₂, IgG₃, or IgG₄ subtypes, IgA, IgE, IgD, or IgM. In one embodiment, the immunoglobulin sequence is obtained from IgG₁ or IgG₃.

Other examples of chimeric heregulin variants include heregulin variants fused to thioredoxin, a "salvage receptor binding epitope," or a cytotoxic polypeptide. Fusion of a heregulin variant with thioredoxin enhances expression and provides a purification handle that facilitates purification using phenylarsine oxide, which can be covalently bound to a solid support, such as agarose. (Agarose functionalized with phenylarsine is available

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commercially as Thibond™ Resin from Invitrogen Corp., San Diego, CA.) Exemplary thioredoxin-variant fusion proteins are described in Example 2.

5 The term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that increases the in vivo serum half-life of the IgG. Salvage receptor binding epitopes suitable for fusion to a heregulin variant according the invention include any of the
10 known salvage receptor binding epitopes.

The term "cytotoxic polypeptide" refers to a polypeptide that inhibits a cellular function or kills cells. Cytotoxic polypeptides suitable for fusion to a heregulin variant include an enzymatically active toxin
15 of bacterial, fungal, plant, or animal origin and fragments thereof and an oncogene product/tyrosine kinase inhibitor, such as a peptide that inhibits binding of a tyrosine kinase to a SH2-containing substrate protein (see WO 94/07913, for example).

20 In one embodiment, a chimeric heregulin variant includes a heregulin variant fused to an enzyme that converts a "prodrug" to an active drug. Typically, the "prodrug" is a precursor or derivative form of a cytotoxic drug that is less cytotoxic than the drug
25 itself and is capable of being enzymatically activated or converted to the cytotoxic drug. The prodrugs of this invention include, but are not limited to, a phosphate-containing prodrug, a thiophosphate-containing prodrug, a sulfate-containing prodrug, a
30 peptide-containing prodrug, a D-amino acid-modified prodrug, a glycosylated prodrug, a β -lactam-containing prodrug, a phenoxyacetamide-containing prodrug, a phenylacetamide-containing prodrug, 5-fluorocytosine, a 5-fluorouridine prodrug, and derivatives thereof.
35 Examples of cytotoxic drugs that can be derivatized to produce a prodrug for use in this invention include,

but are not limited to, those chemotherapeutic agents described above.

A heregulin variant according to the invention is capable of binding an ErbB receptor. The term "ErbB
5 receptor" refers to any of the mammalian class I tyrosine kinase receptors. Examples of such receptors include the ErbB-1 receptor (also known as "the EGF receptor"), the ErbB-2 receptor (also called "the HER2 receptor"), the ErbB-3 (or "HER3") receptor, and the
10 ErbB-4 (or "HER4") receptor. The phrase "capable of binding" is used to describe a polypeptide that binds another polypeptide with a dissociation constant (K_d) of at least 1 mM.

Exemplary heregulin variants that are capable of
15 binding the ErbB-3 and ErbB-4 receptors are discussed above and in the examples. The production of additional heregulin variants, having further modifications (e.g., additional amino acid substitutions, additions, insertions, or deletions, or
20 covalent modifications) and of chimeric heregulin variants is within the level of skill in the art.

Furthermore, in light of the teachings herein, those skilled in the art can design a large number of additional variants that preserve the binding activity
25 of the heregulin variants of the invention. For example, a conservative substitution in a noncritical residue of a heregulin variant (as identified in Example 2) is not expected to significantly alter ErbB receptor binding. Moreover, any effects on ErbB
30 receptor binding can readily be determined in a simple binding assay, such as those described in Examples 1-3. Thus, the invention encompasses all heregulin variants having amino acid substitutions at the specific positions discussed above, regardless of any additional
35 modifications that may be present.

In addition to ErbB receptor binding, a heregulin variant of the invention can possess one or more other

biological activities of a native heregulin. For example, the heregulin variant can also have the ability to activate an ErbB receptor. The phrase "ability to activate an ErbB receptor" refers to the ability to cause the intracellular kinase domain of an ErbB receptor to phosphorylate tyrosine residues. Generally, receptor activation involves binding of a heregulin to a receptor complex of two or more ErbB receptors (e.g., an ErbB-2/ErbB-3 or ErbB-2/ErbB-4 complex). Receptor binding activates a kinase domain of one or more of the receptors, which results in phosphorylation of tyrosine residues in one or more of the receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s). ErbB receptor phosphorylation can be quantified using the tyrosine phosphorylation assays described in Example 3.

Furthermore, a heregulin variant of the invention can be capable of enhancing the survival, proliferation, and/or differentiation of cells having suitable ErbB receptors. The phrase "enhancing survival of cells" refers to increasing the period of existence of cells, either in vitro or in vivo, relative to the period of existence of cells that have not been exposed to the heregulin variant ("untreated cells").

The expression "enhancing proliferation of cells" means increasing the rate or number of mitotic divisions, either in vitro or in vivo, relative to untreated cells. An increase in cell proliferation in cell culture can be detected by counting the number of cells before and after exposure to the heregulin variant, or by microscopic examination of the degree of confluency. Cell proliferation can also be quantified by measuring ³H-thymidine uptake by the cells.

The phrase "enhancing differentiation of cells" refers to increasing the extent of cell specialization. Cell specialization is characterized by the acquisition

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of one or more characteristics that differ from those of the original cells. Thus, the extent of cell specialization is typically determined by screening for a change in the phenotype of the cell (e.g., identifying a change in cellular morphology).

Exemplary cells that express ErbB receptors, and are therefore responsive to heregulins, include SK-BR-3 cells, glial cells, glioblastoma cells, Schwann cells, hepatocytes, epithelial cells, and muscle cells. Glial cells are derived from the central nervous system and include oligodendrocytes and astrocytes. Muscle cells expressing ErbB receptors include muscle cell precursors (myoblasts) as well as the more specialized skeletal, cardiac, and smooth muscle cells.

Other biological activities that a heregulin variant of the invention can possess include induction of ion channel (e.g. Na⁺ channel) formation; induction of acetylcholine receptor synthesis at the neuromuscular junction; enhancement of the formation of a synaptic junction between a neuron and a muscle, nerve, or glandular cell; downregulation of estrogen receptor; and cell internalization (possibly associated with nuclear localization).

A heregulin variant is produced by any suitable method, including peptide synthesis and recombinant techniques. Generally, recombinant techniques, which are described in detail below, are employed for a heregulin variant longer than about 50 or amino acids.

A heregulin variant having enhanced specificity for the ErbB-4 receptor, relative the ErbB-3 receptor, can be prepared by mutagenizing at one or more heregulin residues and selecting variants having enhanced ErbB-4 receptor specificity. Generally, a residue chosen for mutagenesis in this context is characterized by a difference in effect on binding to the ErbB-4 receptor, as compared the effect on binding to the ErbB-3 receptor. Such residues are evident, for

instance, from the alanine-scanning data presented in Example 2.

Variants having enhanced ErbB-4 receptor specificity are selected by screening for binding to ErbB-4 receptor using any suitable screening method, such as monovalent phage display, which described in Example 4. The results can be improved by subjecting variants to "counter-selection," which in this case entails the removal of variants that bind with high affinity to the ErbB-3 receptor. Example 4 demonstrates that counter-selection against ErbB-3-Ig produces a significant enrichment in variants exhibiting greater specificity for ErbB-4-Ig, relative to ErbB-3-Ig, than that of wild-type HRG- β 1.

Nucleic Acid Molecules

The present invention also includes a nucleic acid molecule related to the heregulin variant. The term "nucleic acid molecule" encompasses single-stranded and double-stranded DNA molecules, including genomic DNA, cDNA, DNA produced by an amplification reaction (such as polymerase chain reaction ["PCR"]), and DNA produced by oligonucleotide synthesis, as well as RNA molecules, such as mRNA. Genomic DNA can include non-transcribed and transcribed regions (such as 5' and 3' non-coding regions, introns, and heregulin variant coding regions). cDNA and mRNA molecules contain sequences corresponding to transcribed regions.

A nucleic acid molecule according to the invention has a nucleotide sequence not found in nature and encodes, or is complementary to a nucleic acid molecule encoding, a heregulin variant of the invention or a fragment thereof. A complementary nucleotide sequence is capable of forming Watson-Crick bonds with its complement, in which adenine pairs with thymine or uracil and guanine pairs with cytosine. A double-stranded DNA molecule encodes one of the heregulin

variants, whereas a single-stranded DNA or RNA molecule is either the coding (sense) strand or the noncoding (anti-sense) strand. When the nucleic acid molecule encodes (or is complementary to a nucleic acid molecule encoding) a fragment of a heregulin variant, the
5 fragment includes at least one amino acid substitution.

Because of the redundancy of the genetic code, there are a large number of possible nucleic acid molecules related to each heregulin variant. More
10 specifically, because several different codons encode the same amino acid, a large number of different nucleic acid molecules encode (or are complementary to a nucleic acid molecule encoding) the same heregulin variant.

15 Generally, a heregulin variant of the invention is produced by mutating a naturally occurring DNA sequence to introduce the desired mutations into the heregulin variant amino acid sequence. However, it may also be advantageous to change one or more codons in a nucleic
20 acid molecule without altering the encoded amino acid. Examples of such "silent mutations" within the scope of the present invention include, for example, mutations that create or destroy restriction endonuclease sites to facilitate construction of a desired vector and
25 mutations that enhance expression of the encoded heregulin variant. Examples of the latter include nucleotide substitutions designed to reduce the formation of 5' stem and loop structures in the transcribed mRNA or to provide codons that are more
30 readily transcribed by the selected host (e.g., the well-known preference codons for *E. coli* or yeast expression).

A nucleic acid molecule of the invention can be incorporated into a vector (as described further below)
35 or used, for example, as a hybridization probe or an amplification primer. A hybridization probe according to the present invention is useful for detecting a

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nucleic acid molecule containing a desired mutation, such as, for example, in screening bacterial transformants to identify clones containing the mutated nucleic acid molecule.

5 Such probes are generally at least about 20 nucleotides and usually less than two kilobases. The probe includes a number of nucleotides that is sufficient, under the hybridization conditions used, to hybridize with a mutated sequence to be detected and to
10 be substantially free from hybridization with other sequences. Typically, a probe of the present invention is at least about 50 nucleotides, and usually about 100 nucleotides in length.

 An amplification primer according to the invention
15 can be used in a conventional amplification protocol, such as PCR, to detect a nucleic acid molecule containing a desired mutation or to produce sufficient amounts of such a molecule for sequencing, insertion into a vector, etc. An amplification primer is
20 typically used as a member of a primer pair, including a 5' upstream primer that hybridizes with the 5' end of the nucleic acid sequence to be amplified and a 3' downstream primer that hybridizes with the complement of the 3' end of the sequence to be
25 amplified.

 In general, a primer according to the invention includes a number of nucleotides that is sufficient, under the hybridization conditions used, to hybridize with a mutated sequence and to be substantially free
30 from hybridization with other sequences. The specificity of the primer increases with the number of nucleotides that hybridize with the mutated sequence. In addition, specificity is correlated with the proportion of residues in the primer that hybridize
35 with the mutated sequence. A primer of the present invention generally includes at least about 15 nucleotides, and usually at least about

20 nucleotides. The primer need not exceed about
30 nucleotides, and usually does not exceed about
25 nucleotides. In one variation of this embodiment,
the primer includes between about 20 and about
5 25 nucleotides. Generally, the primers should have a T_m
in the range of about 55°C to about 75°C. In practice,
the T_m is usually between about 60°C to about 65°C to
facilitate amplification under stringent conditions.

10 Vectors and Host Cells

A nucleic acid molecule of the present invention
can be incorporated into a vector for propagation
and/or expression in a host cell. Such vectors
typically contain a replication sequence capable of
15 effecting replication of the vector in a suitable host
cell (i.e., an origin of replication) as well as
sequences encoding a selectable marker, such as an
antibiotic resistance gene. Upon transformation of a
suitable host, the vector can replicate and function
20 independently of the host genome or integrate into the
host genome. Vector design depends, among other
things, on the intended use and host cell for the
vector, and the design of a heregulin variant vector
for a particular use and host cell is within the level
25 of skill in the art.

If the vector is intended for expression of a
heregulin variant, the vector includes one or more
control sequences capable of effecting and/or enhancing
the expression of an operably linked heregulin variant
30 coding sequence. Control sequences that are suitable
for expression in prokaryotes, for example, include a
promoter sequence, an operator sequence, and a ribosome
binding site. Control sequences for expression in
eukaryotic cells include a promoter, an enhancer, and a
35 transcription termination sequence (i.e., a
polyadenylation signal).

The term "operably linked" means that two nucleic acid sequences are in a functional relationship with one another. For example, a promoter (or enhancer) is operably linked to a coding sequence if it effects (or enhances) the transcription of the sequence. A ribosome binding site is operably linked to a coding sequence if it is positioned to facilitate translation. Operably linked nucleic acid sequences are often contiguous, but this is not a requirement. For example, enhancers need not be contiguous with a coding sequence to enhance transcription of the coding sequence.

A heregulin variant expression vector can also include other sequences, such as, for example, nucleic acid sequences encoding a signal sequence or an amplifiable gene. As discussed above, a signal sequence directs the secretion of a polypeptide fused thereto from a cell expressing the chimeric protein. In the expression vector, nucleic acid encoding a signal sequence is linked to a heregulin variant coding sequence so as to preserve the reading frame of the heregulin variant coding sequence. The inclusion of an amplifiable gene (e.g., the dihydrofolate reductase [DHFR] gene) in a heregulin variant expression vector allows selection of host cells containing multiple copies of the nucleic acid molecule encoding the heregulin variant.

A vector of the present invention is produced by linking desired elements by ligation at convenient restriction sites. If such sites do not exist, suitable sites can be introduced by standard mutagenesis (e.g., site-directed or cassette mutagenesis) or synthetic oligonucleotide adaptors or linkers can be used in accordance with conventional practice.

The present invention also provides a host cell containing a vector of this invention. A wide variety

of host cells are available for propagation and/or expression of vectors. Examples include prokaryotic cells (such as *E. coli* and strains of *Bacillus*, *Pseudomonas*, and other bacteria), yeast or other fungal cells, insect cells, plant cells, and phage, as well as higher eukaryotic cells (such as Chinese hamster ovary cells and other mammalian cells). Host cells according to the invention include cells in culture and cells present in live animals, such as transgenic animals.

See U.S. Patent 5,364,934 for more information on vectors and host cells suitable for use in the recombinant production of a heregulin variant.

A vector of the present invention is introduced into a host cell by any convenient method, which will vary depending on the vector-host system employed. Generally, a vector is introduced into a host cell by transformation (also known as "transfection") or infection with a virus (e.g., phage) bearing the vector. If the host cell is a prokaryotic cell (or other cell having a cell wall), convenient transformation methods include the calcium treatment method described by Cohen et al., *PNAS USA* 69:2110-14 (1972), and the polyethylene glycol method of Chung et al., *Nuc. Acids. Res.* 16:3580 (1988). If a prokaryotic cell is used as the host and the vector is a phagemid vector, the vector can be introduced into the host cell by infection, as described in Example 1. Yeast cells can be transformed using polyethylene glycol, for example, as taught by Hinnen, *PNAS U.S.A.* 75:1929-33 (1978). Mammalian cells are conveniently transformed using the calcium phosphate precipitation method described by Graham et al., *Virology* 52:546 (1978), and Gorman et al., *DNA and Protein Eng. Tech.* 2:3-10 (1990). However, other known methods for introducing DNA into host cells, such as nuclear injection, electroporation (see Example 1), and protoplast fusion also are suitable for use in the invention.

In one embodiment, a host cell containing a nucleic acid molecule encoding a heregulin variant is produced by homologous recombination, as described in WO 91/06667. Briefly, this method involves

5 transforming a host cell containing an endogenous heregulin gene with a homologous recombination vector that includes the sequence to be introduced. The homologous recombination vector also includes at least one sequence of at least about 150 nucleotides in
10 length that is homologous with an endogenous sequence flanking the endogenous heregulin gene. Suitable flanking sequences are readily identified, for example, by the method of genomic walking, using a known native heregulin nucleic acid sequence as a starting point.
15 The homologous recombination vector additionally includes an amplifiable gene, such as the DHFR gene.

Transformation is carried out under conditions such that the vector integrates into the host cell genome by recombination. Cells that integrate the
20 vector are then cultured under conditions that select for amplification of the amplifiable gene. The resulting cells are then screened for high levels of heregulin variant production.

25 Recombinant Production of Heregulin Variants

To produce a heregulin variant recombinantly, host cells containing a heregulin variant expression vector are prepared and cultured under conditions suitable for cell growth and for expression of the heregulin
30 variant. In particular, the culture medium contains appropriate nutrients and growth factors for the host cell employed. The nutrients and growth factors required for growth of a selected host cell are, in many instances, well known or can be readily determined
35 empirically by those skilled in the art. Suitable culture conditions for mammalian host cells, for instance, are described in Mammalian Cell Culture

(Mather ed., Plenum Press 1984), and in Barnes and Sato, Cell 22:649 (1980).

In addition, the culture conditions should allow transcription, translation, and protein transport
5 between cellular compartments. Factors that affect these processes are well-known and include, for example, DNA/RNA copy number; factors that stabilize RNA; nutrients, supplements, and transcriptional inducers or repressors present in the culture medium;
10 temperature, pH, and osmolality of the culture; and cell density. The adjustment of these factors to promote expression in a particular vector-host cell system is within the level of skill in the art. Principles and practical techniques for maximizing the productivity of
15 in vitro mammalian cell cultures, for example, can be found in Mammalian Cell Biotechnology: a Practical Approach (Butler ed., IRL Press 1991).

The cell culture procedure employed in the production of a heregulin variant of the present
20 invention can be any of a number of well-known procedures for large- or small-scale production of proteins. These include, but are not limited to, the use of a fluidized bed bioreactor, a hollow fiber bioreactor, a roller bottle culture system, and a
25 stirred tank bioreactor system. A heregulin variant can be produced, for instance, in a batch, fed-batch, or continuous mode process.

Methods for recovery of recombinant proteins produced as described above are well-known and vary
30 depending on the expression system employed. For example, if, as is typical, the heregulin variant is fused to a signal sequence, the heregulin variant is recovered from the culture medium or the periplasm. Conveniently, the variant is secreted into the
35 periplasmic space as a mature protein. The heregulin variant can also be expressed intracellularly and recovered from cell lysates.

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The heregulin variant can be purified from culture medium or a cell lysate by any method capable of separating the variant from components of the host cell or culture medium. Typically the heregulin variant is separated from host cell and/or culture medium components that would interfere with the intended use of the heregulin variant. As a first step, the culture medium or cell lysate is usually centrifuged or filtered to remove cellular debris. The supernatant is then typically concentrated or diluted to a desired volume or diafiltered into a suitable buffer to condition the preparation for further purification.

The heregulin variant is typically further purified in the same manner as the most homologous native heregulin, taking account of any substantial differences in properties between the two molecules. For example, if the heregulin variant is an epitope-tagged heregulin variant, purification can be carried out using an immunoaffinity column containing antibody to the epitope tag. The following exemplary procedures for purifying heregulins can be used or adapted for purifying a heregulin variant of the invention: fractionation on an immunoaffinity column, fractionation on an ion-exchange column, ammonium sulphate or ethanol precipitation, reverse phase HPLC, chromatography on silica, chromatography on heparin Sepharose, chromatography on a cation exchange resin, chromatofocusing, SDS-PAGE, and gel filtration (e.g., using a High Load Superdex 75™ prep grade column).

If the heregulin variant is expressed initially as an insoluble, aggregated form (especially in bacterial host cells), it may be necessary to solubilize and renature the heregulin variant using techniques available in the art for solubilizing and renaturing recombinant protein refractile bodies. See, e.g., U.S. Patent No. 4,511,502.

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In one variation of this embodiment, the heregulin variant is purified (1) to a degree sufficient to obtain at least 15 residues, and preferably 20 residues, of N-terminal or internal amino acid sequence, using a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue stain. As used herein, "homogeneity" means less than about 5% contamination with other source proteins, as determined by staining with Coomassie blue.

Utility of Heregulin Variants

Generally speaking, heregulin variants according to the invention can be used in the same applications as native heregulins. Of course, some heregulin variants within the scope of the invention may be better suited for one application than for other applications. However, those skilled in the art can readily ascertain which heregulin variants are appropriate for a given application by using one or more conventional assays to determine the biological activity of the variants.

Pharmaceutical Compositions and Treatment Methods

Heregulins are useful in treating a wide range of diseases and disorders affecting the nervous system, musculature, and epithelia. In addition, heregulins can be used in the treatment of cancer. As used herein, "treatment" encompasses the treatment of an existing disease or disorder as well as prophylactic measures.

Accordingly, the present invention provides a pharmaceutical composition including a heregulin variant that is useful in treating any of a variety of diseases or disorders. In one embodiment, a pharmaceutical heregulin variant composition is employed to treat a mammal. In particular, the

composition is useful for treating humans, farm animals (e.g., cows and sheep), zoo animals, animals used in sports (e.g., horses), and pets (e.g., dogs and cats). In a variation of this embodiment, the composition is used to treat a human patient.

A heregulin variant according to the invention can be useful in promoting the development, maintenance, and/or regeneration of a neuron in vivo. Neurons that respond to such a variant include central nervous system (brain and spinal chord) neurons, peripheral nervous system neurons (including sympathetic, parasympathetic, sensory, and enteric neurons), and motoneurons. Diseases or disorders amenable to heregulin variant treatment arise in individuals who have suffered nervous system damage due, for example, to trauma, surgery, stroke, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, or a toxic agent.,

A heregulin variant can provide therapeutic benefits to such individuals by promoting the survival, proliferation, or differentiation of neurons. For example, a heregulin variant can be used to promote the survival or proliferation of motoneurons that have been damaged by trauma or surgery. A heregulin variant can also be employed to treat motoneuron disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy or paralysis. In addition, a heregulin variant can also be useful for treating a human neurodegenerative disorder, such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease.

Furthermore, a heregulin variant of the invention can be used to treat neuropathy, especially peripheral neuropathy. As used herein, the term "peripheral neuropathy" refers to a disorder affecting the

peripheral nervous system, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunctions. Examples include, but are not limited to, distal sensorimotor neuropathy and autonomic neuropathies, such as reduced motility of the gastrointestinal tract or atony of the urinary bladder. Peripheral neuropathies amendable to heregulin variant treatment can be inherited, can result from a systemic disease, or can be induced by a toxic agent. Examples of hereditary neuropathies include Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome. Examples of neuropathies associated with systemic disease include post-polio syndrome; and examples of neuropathies induced by a toxic agent include those caused by treatment with a chemotherapeutic agent.

A heregulin variant can also be employed to improve neural function. The beneficial effects of heregulin variant treatment are attributed to induction of the formation of ion channels in cell membranes and enhancement of the formation of synaptic junctions.

A heregulin variant according to the invention can also be used to treat muscle cells and medical conditions affecting muscle cells. In particular, such heregulin variant can be useful for treating muscle damage, decreasing atrophy of muscle cells, and increasing muscle cell survival, proliferation and/or regeneration. Examples of pathophysiological conditions of the musculature amenable to treatment with a heregulin variant include skeletal muscle diseases (e.g., myopathy or dystrophy), cardiac muscle disorders (including atrial cardiac arrhythmias, cardiomyopathy, ischemic damage, congenital disease, and cardiac trauma), and smooth muscle disorders (such as arterial sclerosis, vascular lesion, or congenital

vascular disease). A heregulin variant can also be employed to reduce hypertension and to increase functional acetylcholine receptors on muscle cells (e.g., in individuals having myasthenia gravis or
5 tachycardia).

A heregulin variant of the invention can also enhance repair and/or regeneration of tissues that express ErbB receptors, especially ErbB-2 and either ErbB-3 or ErbB-4 receptors. Accordingly, a heregulin
10 variant can be useful for treating dermal wounds, gastrointestinal disease, Barrett's esophagus, cystic or non-cystic end stage kidney disease, or inflammatory bowel disease. A heregulin variant can also be employed to promote reepithelialization in the human
15 gastrointestinal, respiratory, reproductive, or urinary tract.

In addition, a heregulin variant according to the invention can be useful for inhibiting tumor cell invasion and metastasis. In particular, a tumor
20 characterized by reduced endogenous heregulin levels (Park et al., *Proc. Am. Assoc. Cancer Res.* 34:521 [1993]) is responsive to treatment with a heregulin variant of the invention. Additionally, a tumor that overexpresses ErbB receptors can be treated by using a
25 heregulin variant conjugated to a cytotoxic agent (described above) to direct the cytotoxic agent to the tumor tissue. A heregulin variant-enzyme conjugate can also be employed to target a prodrug (described above) therapy to cells expressing ErbB receptors.

30 A pharmaceutical composition according to the invention is prepared for storage by mixing a heregulin variant having the desired degree of purity with an optional physiologically acceptable carrier, excipient, or stabilizer, such as are described in Remington's
35 Pharmaceutical Sciences 16th Edition (Osol ed., 1980). The composition can be stored in the form of a lyophilized cake or an aqueous solution. A

pharmaceutically acceptable carrier, excipient, or stabilizer is non-toxic to recipients at the dosages employed, and can include a buffer (such as a phosphate buffer, citrate buffer, and buffers made from other organic acids), an antioxidant (e.g., ascorbic acid), a low-molecular weight (less than about 10 residues) polypeptide, a protein (such as serum albumin, gelatin, and an immunoglobulin), a hydrophilic polymer (such as polyvinylpyrrolidone), an amino acid (such as glycine, glutamine, asparagine, arginine, and lysine), a monosaccharide, a disaccharide, and other carbohydrates (including glucose, mannose, and dextrans), a chelating agent (e.g., ethylenediaminetetracetic acid [EDTA]), a sugar alcohol (such as mannitol and sorbitol), a salt-forming counterion (e.g., sodium), and/or a nonionic surfactant (such as Tween™, Pluronic™, and PEG). In one embodiment, the physiologically acceptable carrier is an aqueous pH-buffered solution.

A heregulin variant composition intended for in vivo administration is typically sterile. Sterilization is readily accomplished by filtration through a sterile filtration membrane. If the composition is stored in lyophilized form, the composition can be filtered before or after lyophilization and reconstitution.

A pharmaceutical heregulin variant composition of the invention is generally placed into a container having a sterile access port, such as, for example, an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle.

Methods for administering a pharmaceutical heregulin variant composition do not differ from known methods for administering therapeutic proteins. Suitable routes of administration include, for example, intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes. A pharmaceutical heregulin

variant composition can be administered continuously by infusion or by bolus injection.

If desired, a sustained-release preparation can also be used to administer a heregulin variant. An exemplary sustained-release preparation has a semipermeable matrix of a solid hydrophobic polymer to which the heregulin variant is attached. Examples of suitable polymers include a polyester, a hydrogel, a polylactide, a copolymer of L-glutamic acid and γ -ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, a degradable lactic acid-glycolic acid copolymer, and poly-D-(-)-3-hydroxybutyric acid. Such matrices are in the form of shaped articles, such as films, or microcapsules.

In one embodiment, a sustained-release heregulin variant preparation includes a liposomally entrapped heregulin variant. Liposomes are small vesicles composed of various types of lipids, phospholipids, and/or surfactants. These components are typically arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing heregulin variants are prepared by known methods, such as, for example, those described in Epstein et al., *PNAS USA* 82:3688-92 (1985), and Hwang et al., *PNAS USA* 77:4030-34 (1980). Ordinarily the liposomes in such preparations are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the specific percentage being adjusted to provide the optimal therapy. Useful liposomes can be generated by the reverse-phase evaporation method, using a lipid composition including, for example, phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter.

For treatment of neurologic diseases or disorders, a heregulin variant can be adsorbed onto a membrane, such as a silastic membrane, which can be implanted in proximity to damaged neural tissue, as described in WO 91/04014.

The dosage of a heregulin variant composition to be employed therapeutically depends, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it is necessary for the clinician to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage can range from about 1 $\mu\text{g/kg}$ to up to 100 mg/kg of body weight or more per day, but is typically between about 10 $\mu\text{g/kg/day}$ to 10 mg/kg/day. Generally, the clinician begins with a low dosage of a pharmaceutical heregulin variant composition and increases the dosage until the desired therapeutic effect is achieved.

The administration of a heregulin variant of the invention can be combined with other therapeutic regimens. For the treatment of neurological conditions, a heregulin variant is optionally combined, or administered in concert, with another neurotrophic factor to achieve a desired therapeutic effect. For example, a heregulin variant can be used together with nerve growth factor (NGF), a neurotrophin (e.g., NT-3, -4, or -5), bone-derived nerve factor (BDNF), an insulin-like growth factor (e.g., IGF-1 or IGF-2), gas6, or another neurotrophic factor to achieve a synergistic stimulatory effect on neurons. Suitable dosages for the neurotrophic factors do not differ from those known in the art for such molecules.

For the treatment of cancer, radiation and/or a chemotherapeutic agent can be administered concomitantly with a heregulin variant. Suitable preparation and dosing schedules for such

chemotherapeutic agents are as recommended by the manufacturer or as determined empirically by the clinician. For preparation and dosing schedules for standard chemotherapeutic agents, see Chemotherapy

5 Service (Perry ed., Williams & Wilkins 1992).

Administration of the chemotherapeutic agent can precede, or follow, administration of the heregulin variant, or the chemotherapeutic agent can be given simultaneously therewith. Antibodies against tumor-
10 associated antigens, such as antibodies that bind EGFR, ErbB-2, ErbB-3, or ErbB-4 receptor, or vascular endothelial factor (VEGF) can also be co-administered with an heregulin variant, as can one or more
15 cytokines.

Non-Therapeutic Methods

Heregulin variants according to the invention also be employed in a variety of non-therapeutic applications, such as cell culture methods and
20 diagnostic methods. For example, a heregulin variant can be used to promote the ex vivo survival, proliferation, or differentiation of cells, such as glial, Schwann, and muscle cells. Cultures of such cells are useful for producing cell-specific factors,
25 such as, for example, the nerve growth factor receptor (P75^{NGFR}) which is a Schwann cell-specific factor. Cell-specific factors can be employed directly as diagnostic tools or employed to generate antibodies for diagnostic use.

30 Ex vivo cell cultures can also be used as cellular prostheses for transplantation. For example, Schwann cell cultures can be transplanted into areas of damaged spinal cord to promote regeneration of interrupted central axons or can be used to assist the repair of
35 peripheral nerve injuries.

Accordingly, the present invention provides a cell culture method in which heregulin-responsive cells are

The cells are cultured in the presence of an effective amount of a heregulin variant. The amount of heregulin variant can vary, depending on the cell type and cell culture conditions, but generally is in the range of about 10, ng/ml to about 1 mg/ml. An appropriate concentration for a given cell culture can readily be determined empirically by those skilled in the art.

A heregulin variant can also be employed in the diagnosis of a cancer characterized by *erbB* (e.g., *erbB2*) overexpression. In a diagnostic method according to the invention, a sample is obtained from an individual and contacted with a heregulin variant under conditions that allow specific binding between the variant and any ErbB receptors present in the sample. The sample can be a tissue sample, a bodily fluid sample, or a cell. In the case of a solid tumor, a tissue sample can be taken from a surgically removed

tumor and prepared for testing by conventional techniques. In the case of lymphomas and leukemias, a sample containing lymphocytes, leukemic cells, or lymph tissues is obtained. Other samples, including samples
5 of urine, tear drops, serum, cerebrospinal fluid, feces, sputum, cell extract, and the like, can be useful for diagnosing particular tumors. As used with regard to this method, the term "specific binding" means that the heregulin variant binds an ErbB receptor
10 with an affinity that is sufficiently high that the heregulin variant does not substantially cross-react with other components present in the sample under the suitable reaction conditions.

The amount of heregulin variant that specifically
15 binds to the sample is determined as an indication of ErbB receptor content. For example, a tissue sample can be obtained from a primary tumor and used to prepare formalin-fixed, paraffin-embedded blocks. See Muss et al., *supra*; Press et al., *Cancer Research*
20 54:2771-77 (1994). Tissue sections are then prepared according to known techniques.

A heregulin variant is contacted with a tissue section under conditions that permit specific binding between the variant and ErbB receptors present in the
25 section. Binding is generally detected using a label, such as a radioisotope, a fluorescent label, or an enzyme-substrate labelling system. The label can be conjugated directly to the heregulin variant, as described above.

Alternatively, the label can be bound to the
30 heregulin variant indirectly. For example, the label can be conjugated to an anti-heregulin variant antibody or conjugated to biotin or avidin and used with an anti-heregulin variant antibody conjugated to avidin or
35 biotin (respectively), as described generally in Current Protocols in Immunology (*supra*). Selective

binding between biotin and avidin links the label to the heregulin variant.

While in vitro analysis is normally contemplated, in vivo analysis using a heregulin variant conjugated to a suitable detectable label (e.g., In for imaging) can also be performed. See, e.g., U.S. Patent No. 4,938,948.

A diagnostic method of the invention can be used in combination with other diagnostic/prognostic evaluations such as determining lymph node status, primary tumor size, histologic grade, estrogen or progesterone status, tumor DNA content (ploidy), or cell proliferation (S-phase fraction). See Muss et al., *New Eng. J. Med.* 330:1260-66 (1994).

A heregulin variant according to the invention is also useful as a standard in assays for heregulins (such as a radioimmunoassay, an enzyme-linked immunoassay, and a radioreceptor assay), in an affinity purification technique (e.g., for an ErbB receptor such as ErbB-3 or ErbB-4 receptor), and in a competitive receptor binding assay. A heregulin variant can also be employed as an immunogen for generating anti-heregulin variant antibodies useful in detection and/or purification of heregulin variants.

In addition to the applications discussed above, a heregulin variant that has an enhanced specificity for the ErbB-4 receptor, relative to the ErbB-3 receptor, can be used to bind and stimulate the ErbB-4 receptor preferentially over the ErbB-3 receptor. Such a variant is useful in studies designed to distinguish between the presence of ErbB-3 and ErbB-4 receptors in a sample or to determine whether particular biological actions are mediated by one or both receptors. For instance, in cell types having a relatively small number of ErbB-4 receptors and a large number of ErbB-3 receptors, in the absence of receptor-specific heregulin variants, it is difficult to identify signal

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transduction pathways activated by the different
receptors and to link particular biological actions to
one or both receptors. A variant that preferentially
binds to the ErbB-4 receptor can be used to help
5 elucidate the roles of the ErbB-3 and ErbB-4 receptors
in heregulin action.

Diagnostic Kits and Articles of Manufacture

The present invention also provides kits useful in
10 practicing the above-described methods. In one
embodiment, the invention provides a diagnostic kit,
i.e., a packaged combination of reagents for use in
testing a sample. The components of the kit are
typically provided in predetermined ratios. A kit for
15 detecting ErbB receptors can include, for example, a
heregulin variant labelled with a suitable label or a
heregulin variant with a labelled reagent(s) for
indirect labelling. If the label is an enzyme, the kit
typically includes any substrate or cofactor required.
20 by the enzyme label. Other additives, such as
stabilizers, buffers and the like, can also be included
in the kit. Kit reagents can be provided as dry
powders, usually lyophilized, together with excipients
for preparing kit reagent solutions of the appropriate
25 concentration. Kits also typically include
instructions for carrying out the assay for which the
kit is designed.

The present invention also provides an article of
manufacture containing a pharmaceutical heregulin
30 variant composition useful for the treatment of a
disorder described above. The article of manufacture
includes a container and a label. Suitable containers
include, for example, bottles, vials, syringes, and
test tubes. The container can be made from any of
35 a variety of materials, such as glass or plastic and
can have a sterile access port. The label on, or

associated with, the container indicates the disorder that the composition is to be used to treat.

The article of manufacture can be a component of a kit that includes a second container including a
5 pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. The kit can also include other materials that are desirable from a commercial or user
10 standpoint, such as other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations in the specification are
15 expressly incorporated herein by reference.

EXAMPLE 1

Determination of the Heregulin- β 1 Minimal EGF-Like Domain

20 The smallest portion of HRG- β 1 EGF that provides high-affinity receptor binding in the context of phage display was determined by preparing phagemid vectors that produced HRG- β 1 147-227, 147-244, 177-227, or
25 177-244 fused to the C-terminus of M13 pIII. These HRG- β 1 EGF-like domain fragments were amplified from the vector pHL89 (which is described in Holmes, et al., *Science* 256: 1205-10 [1992]) by PCR with primers having NsiI/XbaI-containing overhangs.

These fragments were inserted into the phagemid
30 display vector pam-g3 by restriction digest-ligation at the same sites to generate constructs pHRG1-g3 (177-227), pHRG2-g3 (177-244), pHRG4-g3 (147-227), and pHRG5-g3 (147-244). pam-g3 was a derivative of phGHam-g3, which was designed for phage display of
35 human growth hormone (hGH) and was described in Lowman et al., *Biochemistry* 30:10832-38 (1991). pam-g3 was produced by removing the hGH gene present in phGHam-g3

and replacing this gene with a stuffer fragment, which provides space for cleavage at the restriction sites used for cloning.

5 pHRG1-g3 contained an Ala227Val mutation that had been introduced in generating the construct. Single-stranded uracil-containing template was produced from this construct and used in site-directed mutagenesis to restore Ala227, generating pHRG6-g3 (177-227). In each of the above-described constructs, the HRG- β 1 EGF-like domain fragment was attached to residue 247 of pIII.

10 In an effort to determine whether the inclusion of an extended flexible linker at the junction between HRG- β 1 EGF and pIII would alleviate potential pIII interference of binding to ErbB-3, two constructs having linkers at this junction were prepared from the pHRG1-g3 template. pHRG8-g3 expressed HRG- β 1 177-228 attached to pIII 323 through a linker containing three consecutive GGGS (SEQ ID NO:34) repeats, and pHRG11-g3 expressed HRG- β 1 177-230 attached to pIII 247 through a

20 GGGSGGG (SEQ ID NO:35) linkage.

The HRG- β 1 EGF-like domains expressed from the above-described constructs are designated herein by removing the "p" and the "-g3" that appear in the name of the construct. Thus, the HRG- β 1 EGF-like domain expressed from the pHRG2-g3 construct is designated

25 "HRG2."

The domains were displayed monovalently on phage as pIII fusion proteins, as described by Bass *et al.*, *Proteins* 8:309-14 (1990), and then analyzed for their binding to the high-affinity ErbB-2/3 receptor-Ig fusion (ErbB-2/3-Ig) using the phage ELISA technique described by Cunningham *et al.*, *EMBO J.* 13:2508-15 (1994), with slight modifications. To produce phage displaying the domains, mutagenesis reaction mixtures

30 were electrotransformed into XL1-BlueTM cells (Stratagene, Inc., La Jolla, CA), according to the manufacturer's protocol. The transformed cells were

35

ErbB receptor-Ig fusions where prepared using vectors that expressed the extracellular domain (ECD) of the ErbB-2, ErbB-3, or ErbB-4 receptor fused to a human IgG constant domain. First, a unique MluI site was engineered into a vector expressing a human IgG heavy chain (pDR2) at the region encoding the hinge domain of the immunoglobulin. MluI sites were also engineered into a set of ErbB expression vectors at the region encoding the ECD/transmembrane junctions of these receptors. All mutagenesis was performed according to the method of Kunkel et al., *Methods Enzymol.* 154:367-82 (1987).

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prk-type plasmid backbone wherein eukaryotic expression was driven by a cytomegalovirus (CMV) promoter.

Receptor fusions were expressed from these constructs, purified, and allowed to form disulfide-linked dimers. Homodimeric ErbB-2, ErbB-3 and ErbB-4 receptor-Ig fusions were produced by transfecting cells with the construct encoding the appropriate receptor fusion. Heterodimeric receptor fusions were generated by co-transfecting two expression vectors encoding different receptor fusions into the same cells. The resulting secreted receptor fusions were mixtures of two types of homodimers and the expected heterodimer.

To express the receptor fusions, adherent HEK-293 cells (ATCC No. CRL1573) were transfected with the appropriate expression vector(s) using the calcium phosphate precipitation method described by Gorman *et al.*, *DNA and Protein Eng. Tech.* 2:3-10 (1990). Serum-containing medium was replaced with serum-free medium at 15 h post-transfection, and the transfected cells were cultured serum-free for 5-7 days.

The resulting conditioned medium was harvested and passed through Protein A columns (1 mL Pharmacia HiTrap™, Piscataway, NJ). Purified receptor fusions were eluted with 0.1 M citric acid (pH 4.2) into tubes containing 1 M Tris-HCl (pH 9.0). The eluted proteins were then dialyzed against PBS and concentrated using Centri-prep-30™ filters (Amicon, Beverly, MA). Glycerol was added to a final concentration of 25%, and the preparations stored at -20°C. The receptor fusion concentration was determined via a Fc-ELISA.

Microtiter plates (Nunc Maxisorp™ 96-well plates, Inter Med, Denmark) for phage ELISA were prepared as follows. The wells were precoated overnight with 0.5 µg of rabbit anti-human IgG (Fc gamma fragment-specific) antibodies (Jackson ImmunoResearch, West Grove, PA) in 100 µL 50 mM NaCO₃ (pH 9.6). The wells were blocked for 30 minutes (min) with 200 µL PBS

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containing 0.1% bovine serum albumin (BSA) and rinsed with wash buffer (PBS containing 0.05% Tween 20™). The wells were then coated with 0.1 µg ErbB-2/3-Ig in binding buffer (PBS, 0.1% BSA, 0.05% Tween 20™) for 1 h, and washed again.

Serial dilutions of soluble ErbB-2/3-Ig (competitor) and a concentration of phage predetermined to give 60% saturation (without competitor) were added to the wells in 100 µL binding buffer. Following incubation for 2 h at room temperature, plates were washed extensively and treated with a 1:900 dilution of anti-M13 horseradish peroxidase conjugate (Pharmacia, Piscataway, NJ) for 20 min. The amount of phage binding was determined by assaying horseradish peroxidase activity using o-phenylenediamine dihydrochloride) substrate solution (Sigma Chemical Company, St. Louis, MO). EC₅₀ values were calculated as the concentration of soluble ErbB-2/3-Ig required to compete half of the phage off the plate.

The results are set forth in Table 3.

Table 3
Initial Heregulin-Phage Variants

Construct	Heregulin-β1 residues	Linker and pIII fusion point	ErbB-2/3-Ig EC ₅₀ (nM)
HRG2	177-244	pIII 247	6.0
HRG4	147-227	pIII 247	38.0
HRG5	147-244	pIII 247	4.7
HRG6	177-227	pIII 247	40.0
HRG7	177-228	pIII 247	42.0
HRG8	177-228 (1-52)	(GGGS) ₃ -pIII 323	11.0
HRG11	177-230	GGGS ₃ GGG-pIII 247	19.0

*Average of duplicate experiments

All phage stocks bound specifically to immobilized ErbB-2/3-Ig and could be competed off with similar EC₅₀ values (5-42 nM). These values were about 100-fold higher than the previously measured dissociation

In any event, the HRG- β 1 147-176 did not appear to
10 enhance the binding of phage displaying the HRG- β 1
EGF-like domain to ErbB-3-Ig, whereas HRG- β 1 228-244
contributed slightly to binding affinity. Thus, the
minimal EGF-like domain of HRG- β 1 was defined as
HRG- β 1 177-228.

EXAMPLE 2

This example describes the identification of active residues in the heregulin- β 1 (HRG- β 1) EGF-like domain (HRG- β 1 177-229) that play a role in the binding of HRG- β 1 to the ErbB-3 and ErbB-4 receptors. Active residues were identified by mutating individual amino acids in this domain to alanine. The mutated domains (hereinafter "variants") were displayed monovalently on

phage as pIII fusion proteins and variant affinities for ErbB-3 and ErbB-4 were determined by phage ELISA. Selected variants were expressed as thioredoxin fusion proteins, which were also assayed for ErbB-3 and ErbB-4 affinity.

Alanine Scanning Mutagenesis and Phage Display

Alanine-substituted variants were generated by site-directed mutagenesis according to Kunkel et al., *Methods Enzymol.* 154:367-82 (1987) (hereinafter "Kunkel mutagenesis"), using uracil-containing single-stranded DNA template prepared from pHRG2-g3. pHRG2-g3, which is described in Example 1, expressed HRG- β 1 177-244 fused to pIII 247. A series of oligonucleotides was used to generate a series of constructs that expressed a series of variants in which consecutive residues were mutated to alanine. Phage stocks were prepared from these constructs as described in Example 1, except that PEG(8000) was used to precipitate the phage. The affinities of the alanine-substituted variants for ErbB-3-Ig and ErbB-4-Ig was determined by phage ELISA as described in Example 1. The results are shown in Figure 2, which indicates the ratio of the EC_{50} for each variant compared to the EC_{50} for wild-type HRG- β 1 177-244, also displayed on phage. In this plot, a ratio of one indicates that there was no difference in affinity for variant binding compared to wild-type HRG- β 1 177-224, and a ratio of, e.g., five indicates that the variant bound the receptor with an affinity five-fold less than that of wild-type HRG- β 1 177-224.

Expression, Purification, and Assay of Soluble Alanine-Substituted Variants

A number of alanine-substituted variants were expressed in soluble form as thioredoxin (Trx) fusion proteins. To prepare suitable expression vectors, a Trx expression vector was first generated from pET23a

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(Novagen, Inc., Madison, WI). pET23a was digested with NdeI (which cuts at base 238) and HindIII (which cuts at base 173), and a fragment encoding Trx was inserted. This fragment was obtained from pTrxFus (bases 2722-3180; Invitrogen Corp., San Diego, CA). The NdeI site, which includes the Trx translation start site, was then destroyed by cutting with NdeI and religating with Klenow. This removed the NdeI site, while retaining the Trx translation start.

10 Vectors encoding HRG- β 1 alanine-substituted variants were initially generated by Kunkel mutagenesis in a pRK5.gDhrgB1 vector (described in Gorman et al., DNA Prot. Eng. Tech. 2:2-10 [1990]). The sequences encoding the variants could be cleaved from these
15 vectors using NdeI and BamHI. To facilitate cloning of such fragments into the Trx expression vector, Kunkel mutagenesis was used to engineer a KpnI site into the pRK5.gDhrgB1 vector encoding wild-type HRG- β 1 146-244 immediately upstream of the NdeI site (at base 5407).
20 A KpnI-BamHI fragment encoding wild-type HRG- β 1 146-224 was then cleaved from pRK5.gDhrgB1 and inserted to the Trx expression vector at KpnI and BamHI cloning sites at the 3' end of the sequence encoding Trx. This introduced an NdeI site immediately downstream of the
25 KpnI site. In the resultant vector, the wild-type HRG- β 1 sequence could be removed by digesting with NdeI and BamHI and replaced with an NdeI-BamHI fragment encoding a variant. The series of Trx-variant expression vectors thus obtained expressed Trx-variant
30 fusions that contained an enterokinase protease recognition site (DDDDK; SEQ ID NO:37) between the Trx and the variant sequences.

Expression of Trx-variant fusion proteins was driven by the inducible T7 promoter from pET23a.
35 Cloning, cell growth, and expression were carried out as described in the Novagen pET system manual. Briefly, cloning was done in XL1-Blue™ cells

Cells were lysed by freezing on dry ice, thawing at 37°C, followed by vigorous sonication. The freeze thaw, and sonication cycle was repeated three times. Protein was further solubilized in 6 M GdHCl, 0.1 M Tris-HCl (pH 8.8), sulfitolized by the addition of 0.1 M Na₂SO₃, 0.2 M Na₂S₄O₆, and stirred at room temperature for 1.5 h. Protein was dialyzed into 0.05 M Tris-HCl (pH 7.5), 0.01 M methionine. After dialysis, the insoluble material was removed by centrifugation at 35K x g for 15 min.

The affinities of the Trx-variant fusions for ErbB
35 receptor-Ig fusions were determined by measuring
inhibition of ¹²⁵I-HRG-β1 177-244 binding to ErbB-3-Ig
and ErbB-4-Ig. Receptor fusions were coated on plates

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(Nunc Maxisorp CTM break-apart strip wells, Inter Med, Denmark) via anti-human IgG, as described in Example 1 for phage ELISA. Binding assays were carried out with a constant amount of ¹²⁵I-HRG- β 1 177-244 (100-300 pM) and varying concentrations (100 pM - 4 μ M) of unlabeled Trx-variant fusion. Following incubation for 1-3 h at room temperature, plates were washed, and the amount of bound ¹²⁵I-HRG- β 1 177-244 in each well was counted on a gamma counter (Isodata, ICN Biomedic Systems, Huntsville, AL). For the ErbB-3 binding assays, the blocking buffer was TBST (0.025 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.02% Tween 20TM) containing 1% BSA; the binding buffer was RPMI 1640TM cell culture media (Gibco-BRL, Gaithersburg, MD) containing 2 mM glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES buffer (pH 7.2), 0.2% BSA; and the wash buffer was TBST. For the ErbB-4 binding assays, PBS containing 1% BSA, was used as the blocking and binding buffers, and the wash buffer was PBS containing 0.05% Tween 20TM.

The results are shown in Tables 4 and 5.

5

	<u>Variant</u>	<u>Phage EC₅₀ (nM)</u>	<u>Sol EC₅₀ (nM)</u>
	HRG-β1 [#]	13	9
	TH-WT [®]		47.6
10	S177	67	129
	H178	>1000	476
	L179	~1000	394
	F189	892	975
	N192	>1000	1642
15	G193	>1000	26.9
	G194	500	1212
	E195	>1000	42
	V199	27	
	K200	20	45.4
20	P205	22	39.5
	R207	>1000	245
	K211	>1000	248
	E215	51	156
	F216	500	2550
25	T217	>1000	145
	G218	>1000	668
	R220	>1000	no binding
	Y224	865	339
	F229	6	177

30

*Variants are identified by the HRG- β 1 residues mutated to alanine.

35

*HRG-B1 is recombinantly produced HRG-β1 177-244.

*TH-WT is thioredoxin fused at its C-terminus to wild-type HRG- β 1 146-244.

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Table 5
EC₅₀ Values for Phage and Soluble Alanine-Substituted
Variant Binding to the ErbB-4 Receptor

5

	<u>Variant*</u>	<u>Phage EC₅₀ (nM)</u>	<u>Sol EC₅₀ (nM)</u>
	HRG-β1 [#]	19	14.8
	TH-WT [®]		15.4
10	S177	83	54.6
	H178	32	138
	L179	56	51.7
	F189	106	565
	N192	188	696
15	G193	23	76.3
	G194	65	275
	E195	24	13.2
	V199	47	246
	K200	25	16.9
20	P205	8	34.2
	R207	30	24.3
	K211	59	124
	E215	32	104
	F216	>1000	173
25	T217	14.2	32.7
	G218	>1000	608
	R220	>1000	no binding
	Y224	51	24.5
	F229	>1000	89.9

30

*Variants are identified by the HRG-β1 residues mutated to alanine.

35

[#]HRG-B1 is recombinantly produced HRG-β1 177-244.

[®]TH-WT is thioredoxin fused at its C-terminus to wild-type HRG-β1 146-244.

Selection of Heregulin- β 1 EGF Domain Variants Using Monovalent Phage Display

Variants of HRG- β 1 EGF were prepared and selected for binding to ErbB-3-Ig using monovalent phage display, according to the method of Bass et al., *Proteins* 8:309-14 (1990). As discussed in detail below, an HRG- β 1 EGF phagemid vector was prepared, in which HRG- β 1 EGF was fused to a C-terminal fragment of the M13 coat protein pIII. Kunkel mutagenesis was performed to introduce stop codons into this vector at sites selected for randomization. This step ensures that the starting vector is incapable of expressing the wild-type polypeptide. Stretches of four to six residues per library were randomized in a linear fashion, except for the six cysteines, Phe189 (HRG- β 1 EGF Phe13) and the two most C-terminal residues (see Figure 3). Phe189 was not altered because this residue is conserved as an aromatic residue in EGF and TGF- α and forms a stacking interaction with Tyr208 (HRG- β 1 EGF Tyr32) Jacobsen et al., *Biochemistry* 35:3402-17 (1996). HRG- β 1 EGF was thus covered in eight libraries, designated A-E, G, H and I.

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ErbB-3-Ig with an affinity similar to that of wild-type (Table 13).

5 An additional library (F) was created to randomize a surface patch composed of side chains from the first and second β -sheets, which included HRG- β 1 178, 180, 198, and 200 (HRG- β 1 EGF 2, 4, 22, and 24).

10 The selected sites in the starting vectors were randomized by Kunkel mutagenesis to produce HRG- β 1 EGF libraries. Phage displaying mutated HRG- β 1 EGFs were produced from the libraries under conditions such that, statistically, each phage particle displayed no more than one copy of the mutated HRG- β 1 EGF. See Bass et al., supra. These phage were then selected for binding to (sorted against) ErbB-3-Ig immobilized on an
15 ELISA plate. Bound phage were eluted and used to reinfect host cells, which were used to produce new phage for another round of sorting. This process was repeated six to seven times for each library. Twelve clones from the phage selected from each library were
20 then sequenced.

Construction of Phage Libraries

Phage libraries were constructed by Kunkel mutagenesis using uracil-containing single-stranded DNA
25 template prepared from pHRG8-g3. pHRG8-g3, which is described in Example 1, expressed HRG- β 1 177-228 (HRG- β 1 EGF 1-52) fused via a linker to pIII residue 323. For each library, TAA and TGA stop codons were installed at positions selected for randomization
30 to generate custom templates that eliminated wild-type background from the pools. Positions were fully randomized by mutation to NNS codons (where N is any of the four bases and S is either G or C). One oligonucleotide was used for each library mutagenesis
35 reaction except for Library F, for which two oligonucleotides (one randomizing HRG- β 1 178 and 180 [HRG- β 1 EGF 2 and 4] and the other randomizing HRG β -1

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198 and 200 [HRG- β 1 EGF 22 and 24]), were used simultaneously. Mutagenesis oligonucleotides contained 18-base overhangs on either side of the randomized residues.

5 Mutagenesis reaction mixtures were electro-transformed into XL-1 blue cells (Stratagene, Inc., La Jolla, CA), according to the manufacturer's protocol. The transformed cells were then infected with 10^{11} pfu M13K07 helper phage (Promega Corp.,
10 Madison, WI), and phage stocks (about 10^{14} phagemid/mL) were prepared as described in Example 1.

Between 1.0×10^8 and 6.4×10^8 transformants were obtained for each library, meaning that the libraries containing five or fewer randomized codons had
15 excellent representation of the possible amino acid sequence combinations (3.36×10^7 possible DNA sequences; 3.2×10^6 possible amino acid sequences). Library B, containing six randomized codons (1.1×10^9 DNA sequences), had 4.8×10^8 total transformants.

20

Selection of Phage for ErbB-3-Ig Binding

Monovalent phage were prepared and the selection performed on ErbB-3-Ig prebound to microtiter plates via capture with polyclonal antibodies to the human Fc
25 fragment, as described in Example 1. Approximately 10^{12} phage in 100 μ L binding buffer (PBS, 0.1% BSA, 0.05% Tween 20™) were applied to an ErbB-3-Ig-coated well and a control well to which no ErbB-3-Ig had been added. Following a 2 h incubation at room temperature, the
30 plates were washed extensively (12x) and phage eluted by adding 100 μ L of a solution of 50 mM HCl and 0.05% Tween™ 20 and shaking for 10 min.

Eluates were neutralized with 10 μ L 1 M Tris-HCl (pH 8.0) and 20 μ L used for titration on log-phase XL-1
35 blue cells. The remainder was used to infect 1 mL of log-phase XL-1 blue cells (30 min at 37°C), which were then superinfected with 2×10^{10} pfu M13K07 phage and

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grown in 25 mL 2YT broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) containing 50 μ g/mL carbenicillin for 18-24 h. Phage were harvested as described above and the cycle repeated. The libraries enriched rapidly, such that by round six of selection the ratio of phage eluted from positive (ErbB-3-Ig-coated) wells to negative (anti-hu Fc precoat only) wells was between 39 and 9200.

After round six (libraries A, B, D-F) or round seven (libraries C, G-I) of selection, twelve clones from each library were randomly picked and sequenced by the dideoxy method. See Sanger et al., *PNAS USA* 74:5463-67 (1977). The amino acids at the randomized positions deduced from the DNA sequences are shown in Tables 6-12 (a "." indicates a residues that is identical to the wild-type residue). The consensus selected residues at each position are displayed graphically in Figure 3. In general, there were a large number of mutations, in some cases with dramatic changes in the character of the side chains. At several positions that sorted to a particular residue, a mixture of DNA codons was found, providing confidence that the libraries had large diversity and that selection was at the protein level. In several of the libraries, there was a spontaneous mutation of HRG- β 1 Met226Ile (HRG- β 1 EGF Met50Ile) due to a one-base change in this codon. This mutation results in a significant affinity enhancement for ErbB-3-Ig binding. The sequencing results for each library are summarized below.

Library A - HRG- β 1 177-181 (HRG- β 1 EGF 1-5)

HRG- β 1 177-181 (HRG- β 1 EGF 1-5) is present in the first β -strand in the wild-type N-terminal subdomain. The amino acid changes in the variants selected from this library are shown in Table 6.

Table 6
Library A Variants

		Position in HRG-β1				
	Variant No.	177	178	179	180	181
5	Wild-type	S	H	L	V	K (SEQ ID NO:38)
	1	W	R	.	.	P (SEQ ID NO:39)
	2	W	S	.	Q	P (SEQ ID NO:40)
	3, 5, 10	W	E	.	.	P (SEQ ID NO:41)
	4	W	S	.	.	. (SEQ ID NO:42)
10	6	W	S	.	I	P (SEQ ID NO:43)
	7	W	R	.	.	A (SEQ ID NO:44)
	8	W	A	.	.	P (SEQ ID NO:45)
	9	W	S	.	Q	. (SEQ ID NO:46)
	11	W	E	.	.	A (SEQ ID NO:47)
15	12	W	S	.	E	P (SEQ ID NO:48)

Upon randomization, Ser177 (Ser1) sorted exclusively to Trp. His178 (His2) sorted to mixed hydrophilic residues, but the wild-type residue was not among them. Leu179 (Leu3) was conserved in all variants sequenced. Val180 (Val4) sorted to wild-type in eight out of 12 variants, and the remaining variants had conservative substitutions at this position, with the exception of a Val180Glu (Val4Glu) mutation. At Lys181 (Lys5), Pro appeared in eight variants, and the wild-type residue was found in two variants.

Four variants from library A did not contain amino acid substitutions at positions randomized in library A. Instead, these variants contained amino acid substitutions at positions randomized in library B, and thus these variants are listed as variants B1-B4 in Table 7. Similarly, four variants from library B did not contain amino acid substitutions at positions randomized in library B, but rather contained substitutions at positions randomized in

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library A. These variants are listed as variants A5-A8 in Table 6.

5 *Library B - HRG-β1 183-188 (HRG-β1 EGF 7-12)*

HRG-β1 183-188 (HRG-β1 EGF 7-12) has a helical character in the wild-type protein. The amino acid changes in the variants selected from this library are shown in Table 7.

10

Table 7
Library B Variants

		Position in HRG-β1					
15	Variant No.	183	184	185	186	187	188
	Wild-type	A	E	K	E	K	T (SEQ ID NO:49)
	1*	G	V	G	R	D	G (SEQ ID NO:50)
	2*	G	G	E	R	E	G (SEQ ID NO:51)
20	3	G	.	E	R	E	G (SEQ ID NO:52)
	4*, 5*	G	W	D	R	E	G (SEQ ID NO:53)
	6*	G	V	Q	R	E	G (SEQ ID NO:54)
	7	G	.	E	R	A	G (SEQ ID NO:55)
	8	G	K	E	R	E	G (SEQ ID NO:56)
25	9*	T	N	S	R	E	G (SEQ ID NO:57)
	10*	D	K	S	R	E	G (SEQ ID NO:58)
	11*	G	.	D	R	.	Q (SEQ ID NO:59)
	12	G	R	E	R	E	G (SEQ ID NO:60)

30 *Variant also contained Met226Ile.

Randomization of this region produced the most dramatic changes from the wild-type sequence, although the generally hydrophilic character of this region was maintained in the variants sequenced. In particular, this six-residue stretch sorted to Gly residues at the first and last positions, Ala183 and Thr188 (Ala7 and Thr12). There was also a change in registry of

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positive and negative charges at Lys185 (Lys9), which sorted to Glu and Asp, among others; Glu186 (Glu10), which sorted exclusively to Arg; and Lys187 (Lys11), which sorted to Glu and Asp. Glu184 (Glu8) sorted to a variety of different types of residues, indicating that this side chain does not play an important role in ErbB-3 receptor binding.

Library C - HRG- β 1 191-195 (HRG- β 1 EGF 15-19)
 HRG- β 1 191-195 (HRG- β 1 EGF 15-19) includes the β -turn between the helix and the second β -strand. The amino acid changes in the variants selected from this library are shown in Table 8.

Table 8
 Library C Variants

Variant No.	Position in HRG- β 1				
	191	192	193	194	195
Wild-type	V	N	G	G	E (SEQ ID NO:61)
1, 2, 4, 5, 7-12	(SEQ ID NO:62)
3	V (SEQ ID NO:63)
6	Q (SEQ ID NO:64)

Randomization and selection produced variants in which the wild-type amino acid sequence in this region was almost completely conserved. Mutations were found in only two variants, both at Glu195 (Glu19). This result is consistent with an important role for the wild-type residues in this region in ErbB-3 receptor binding.

Library D - HRG- β 1 197-201 (HRG- β 1 EGF 21-25)

HRG- β 1 197-201 (HRG- β 1 EGF 21-25) is present in the second β -strand in the N-terminal subdomain. The amino acid changes in the variants selected from this library are shown in Table 9.

Table 9
Library D Variants

		Position in HRG- β 1				
Variant No.		197	198	199	200	201
	Wild-type	F	M	V	K	D (SEQ ID NO:65)
	1*, 2*, 8*, 12*	Y	K	.	R	I (SEQ ID NO:66)
15	3	.	R	.	.	T (SEQ ID NO:67)
	4, 5, 7, 9	Y	R	.	.	T (SEQ ID NO:68)
	6	Y	.	I	.	Y (SEQ ID NO:69)
	10	Y	.	.	.	T (SEQ ID NO:70)
20	11	M	R	.	R	T (SEQ ID NO:71)

*Variant also contained Met226Ile.

Randomization in this region yielded either a gain or loss of charge for two of the five residues. Phe197 (Phe21) sorted to Tyr in 10 out of 12 variants, maintaining aromaticity at this position. Met198 (Met22) sorted to a positively charged residue in 11 out of 12 variants. Val199 (Val23) was conserved in all variants, and Lys200 (Lys24) sorted either to wild-type or to Arg, retaining the positive charge at this position. Asp201 (Asp25) sorted to uncharged residues Thr or Ile, retaining the β -branch character of this position. Variants D1, D2, D8, D12 also included the spontaneous affinity-enhancing Met226Ile (Met50Ile) mutation.

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Library E - HRG- β 1 205-209 (HRG- β 1 EGF 29-33)

HRG- β 1 205-209 (HRG- β 1 EGF 29-33) includes residues present in the third β -strand in the N-terminal subdomain. The amino acid changes in the variants selected from this library are shown in Table 10.

Table 10
Library E Variants

10

		Position in HRG- β 1				
Variant No.		205	206	207	208	209
Wild-type		P	S	R	Y	L (SEQ ID NO:72)
15	1	T	P	Y	L	M (SEQ ID NO:73)
	2, 4	Y	G	Y	L	M (SEQ ID NO:74)
	3*	Y	R	Y	R	M (SEQ ID NO:75)
	5, 12	T	H	Y	R	G (SEQ ID NO:76)
	6	T	H	Y	R	M (SEQ ID NO:77)
20	7*	Y	K	Y	R	M (SEQ ID NO:78)
	8, 9	T	K	Y	R	G (SEQ ID NO:79)
	10	Y	K	Y	R	. (SEQ ID NO:80)

11#

25

*Variant also contained Met226Ile.

#Variant E11 was a contaminant from library F (identical to the other 12 library F variants).

30

Upon randomization, Pro205 (Pro29) sorted to Thr or Tyr. Ser206 (Ser30) sorted to mixed residues, predominantly those having basic side chains, although Gly also appears twice (in sequences derived from the same variant). An inversion of side chains occurred for Arg207 (Arg31) and Tyr208 (Tyr32), the first of which sorted exclusively to Tyr and the second of which sorted primarily to Arg (seven variants) and Leu (four variants). This finding was particularly unexpected, given that Tyr208 stacks with Phe189 (Phe13) in the

40

structure and is conserved in the EGF sequence. See Jacobsen et al., *Biochemistry* 35:3402-17 (1996). At Leu209 (Leu33), the relatively conservative Met substitution was found in the majority of variants, but Gly was also found.

Library G - HRG- β 1 211-216 (HRG- β 1 EGF 35-40)

HRG- β 1 211-216 (HRG- β 1 EGF 35-40) includes the first β -strand of the C-terminal subdomain of HRG- β 1 EGF. The amino acid changes in the variants selected from this library are shown in Table 11.

Table 11
Library G Variants

Variant No.	Position in HRG- β 1					
	211	212	213	214	215	216
Wild-type	K	C	P	N	E	F (SEQ ID NO:81)
1, 5, 6, 10, 12	R	.	S	L	.	(SEQ ID NO:82)
2	R	.	S	E	.	(SEQ ID NO:83)
3	.	.	.	K	.	M (SEQ ID NO:84)
4	R	.	T	V	.	Y (SEQ ID NO:85)
7, 8	R	.	T	V	.	Y (SEQ ID NO:86)
9	.	.	N	S	.	(SEQ ID NO:87)
11	R	.	K	K	.	(SEQ ID NO:88)

Upon randomization, 10 out of 12 variants contained a Lys211Arg (Lys35Arg) mutation, thus retaining a positive charge at this position, which lies between two cysteines. Pro213 (Pro37) sorted to mixed hydrophilic residues, and Asn214 (Asn38) sorted to a mixture of residues, with Leu and Val appearing most frequently. Glu215 (Glu39) was conserved in all variants, and Phe216 (Phe40) was retained in eight out of 12 variants with a conservative Tyr substitution in three of the remaining variants.

Library H - HRG- β 1 217-220 (HRG- β 1 41-44)

The HRG- β 1 217-220 (HRG- β 1 EGF 41-44) library proved vulnerable to contamination by a high-affinity variant from library B (variant B5). This variant was found in 11 out of 12 variants. In the single unaffected variant, the wild-type amino acid sequence was conserved except for an Asp219Glu (Asp43Glu) mutation. This result suggests that this region requires the wild-type or similar sequences for optimal binding.

Library I HRG- β 1 222-226 (HRG- β 1 EGF 46-50)

HRG- β 1 222-226 (HRG- β 1 EGF 46-50) includes a short strand of β -sheet that aligns with the strand of β -sheet at HRG- β 1 213-216 (HRG- β 1 EGF 38-40). The amino acid changes in the variants selected from this library are shown in Table 12.

Table 12
Library I Variants

Variant No.	Position in HRG- β 1				
	222	223	224	225	226
Wild-type	Q	N	Y	V	M (SEQ ID NO:89)
1, 4, 8	.	W	.	.	I (SEQ ID NO:90)
2, 3, 5-7, 9-12	.	H	.	.	I (SEQ ID NO:91)

Upon randomization of HRG- β 1 222-226, only two types of variants were found in the 12 sequenced, both having wild-type residues conserved at Gln222 (Gln46), Tyr224 (Tyr48), and Val225 (Val49), and both having a Met226Ile (Met50Ile) mutation. Asn223 (Asn47) sorted to His (nine variants) or Trp (three variants). The strong affinity-enhancing effect of Met226Ile is evidenced by its presence in all variants sequenced from this library and a high frequency of occurrence in variants from several other libraries.

Library F - HRG- β 1 178, 180, 198, and 200 (HRG- β 1 EGF 2, 4, 22 and 24)

When His178 (His2), Val180 (Val4), Met198 (Met22), and Lys200 (Lys24) were simultaneously randomized, only one type of variant was found. His178, Val180, and Lys200 sorted to wild-type residues, and Met198 sorted to Lys. These variants additionally contained the spontaneous Met226Ile (Met50Ile) mutation, which gave the variants a significant selective advantage over other sequences. It is striking that the wild-type His was found at position 178 because none of the 12 variants sequenced from library A contained His178.

Conservation of HRG- β 1 EGF Residues in Phage Display and Alanine Scanning Results

Positions where alanine substitution strongly affected binding affinity tended to sort to the wild-type residue. See, for example, the data for positions at the junction between the N- and C-terminal subdomains, (i.e., the β -turn at HRG- β 1 191-195 [HRG- β 1 EGF 15-19] and the loop at HRG- β 1 217-220 [HRG- β 1 EGF 41-44]) Additionally, positions where alanine substitution produced less significant effects tended to undergo substantial mutation upon phage display, as seen for the helical stretch at HRG- β 1 183-188 (HRG- β 1 EGF 7-12).

Analysis of the Impact of Selected Mutations on Receptor Affinity and Specificity

Individual variants from each library were chosen for phage production and further characterization of the mutated HRG- β 1 EGFs ("variants") displayed on the phage. The choice of variants for further characterization was based on selection frequency, with a bias towards sequences not containing the advantageous Met226Ile (Met50Ile) substitution. In addition, phagemid vectors for use in producing phage

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*Based on the wild-type HRG- β 1 EGF-phage EC₅₀
15 (=135 \pm 104 nM for ErbB-3-Ig, 163 \pm 112 nM for
ErbB-4-Ig) determined on during the same assay run.
Shown are standard deviations for averages of 2-4
duplicate runs, or the average only for assays
performed in duplicate for one run.

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25 Variants from library A had EC₅₀ values very similar to wild-type HRG-β1 EGF. Variants from libraries B and D had significantly enhanced affinity for ErbB-3-Ig and ErbB-4-Ig, in the range of three to five times the wild-type affinity for selected variants not containing Met226Ile (Met50Ile). (See variants B3 and D4.) Substantially greater enhancements in affinity (up to 26-fold wild-type HRG-β1 EGF) were measured for variants that contained Met226Ile (Met50Ile). (See variants B5, B10, and D1).

Variants from library E showed even greater
 affinity enhancements. For example, variant E2, which
 differed from the library consensus sequence (Figure 3)
 at HRG- β 1 206 and 208 (HRG- β 1 EGF 30 and 32), had an
 35 affinity enhancement of 28-fold. The effects appear to
 be associated with the amino acid substitutions at

HRG- β 1 205-209 (HRG- β 1 EGF 29-33) rather than the adjacent three-residue deletion, since the affinity for ErbB-3-Ig of a Δ 202-204 (Δ 26-28) control variant (HRG63) was similar to that of wild-type HRG- β 1 EGF (Table 13).

The single variant from library F, having Met198Lys (Met22Lys) and Met226Ile (Met50Ile) substitutions, showed an enhancement only slightly above that for Met226Ile alone, indicating a small effect attributable to the Met198Lys mutation. Variants from library G showed little, if any, ErbB-3-Ig affinity enhancement. The single Asp219Glu (Asp43Glu) mutation from the lone library H variant provided a modest increase in affinity. The two library I variants had significant enhancements in affinity for ErbB-3-Ig and ErbB-4-Ig. The enhancements were attributable to an approximately six-fold effect from the Met226Ile (Met50Ile) mutation (see variant HRG90), and an additional enhancement of about two- to three-fold from Asn223 (Asn47) to Trp or His mutations.

Phage displaying combination variants containing variant A3 mutations performed poorly. The A3 + B3 combination had an EC₅₀ for ErbB-3-Ig similar to that of variant B3, but the other combinations tested did not bind with detectable affinity. This could be due to a disadvantageous interaction of the Ser177Trp, His178Glu, or Lys181Pro (Ser1Trp, His2Glu, or Lys5Pro) mutations with mutations from the other libraries. The combination of the B3 and E2 mutations yielded slightly diminished affinities relative to that of variant E2.

The combination of mutations from variants from the other libraries gave closer to additive behavior and resulted in the enhancement of EC₅₀ values by greater than 50-fold. The EC₅₀ values for the best combination variants were close to the lower limit of the assay at the receptor coating concentration used (approximately 4 nM). Using lower levels of receptor

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Expression, Purification, and Assay of Soluble HRG- β 1 EGF Variants

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Wild-type HRG- β 1 EGF and variants thereof were purified to homogeneity from periplasmic shockates in a single reverse-phase HPLC step. Briefly, cells were harvested after 24 h of growth at 30°C ($OD_{550} = 1.2$) by centrifugation at 4500 rpm, and the pellets were frozen in ethanol/dry ice for 2 h. Following resuspension and thawing in 5 mM $MgCl_2$, 75 mM $CaCl_2$, 1 mM phenyl methyl sulfonyl fluoride, and 10 mM Tris-HCl (pH 7.6), shocked cells were removed by centrifugation, leaving shockates. Shockates were filtered and chromatographed by semipreparative C18 reverse-phase HPLC using a gradient from 0-40% acetonitrile over 80 min, with a flow rate of 3 mL/min. Fractions shown by electrospray mass spectrometry to contain HRG- β 1 EGF (or variants thereof) were lyophilized and resuspended in 1 mM EDTA, 10 mM Tris-HCl (pH 7.6). Proteins were found to be essentially homogeneous as determined by amino acid analysis and SDS-PAGE.

The affinities of the soluble variants for ErbB-Ig were determined by measuring inhibition of ^{125}I -HRG binding to ErbB-2/3, -3 and -4 receptor-Ig fusions, as previously described in Example 1. Although the ErbB-2/3-Ig preparation also contains ErbB-2/2 and 3/3 homodimers (as a result of coexpression of ErbB-2- and -3-Ig's), the displacement of ^{125}I -HRG should be predominantly from ErbB-2/3-Ig because of the approximately 100-fold higher affinity of wild-type HRG- β 1 EGF for the 2/3-heterodimer versus the 3/3 homodimer.

The results are shown in Table 14.

Table 14
*Binding and Activation Parameters for Soluble
 Heregulin- β 1 EGF Variants*

Construct	ErbB-3-Ig EC ₅₀ (nM)	ErbB-4-Ig EC ₅₀ (nM)	ErbB-2/3-Ig EC ₅₀ (nM)	KIRA EC ₅₀ (nM)
HRG8 (WT)	2.3 \pm 0.2	1.5 \pm 0.1	0.033 \pm 0.003	0.212
A3	4.3 \pm 0.2	2.1 \pm 0.1	0.067 \pm 0.007	0.182
E2	2.0 \pm 0.2	2.4 \pm 0.02	0.035 \pm 0.007	0.092
F1	0.60 \pm 0.04	0.26 \pm 0.011	0.061 \pm 0.002	0.151
HRG58	0.80 \pm 0.01	0.30 \pm 0.005	0.075 \pm 0.006	0.129
HRG73	2.7 \pm 0.1	0.31 \pm 0.003	0.20 \pm 0.006	0.173

The variants showed higher affinity for ErbB-3-Ig than did wild-type HRG- β 1 EGF (up to 4-fold), although the affinity enhancements were less dramatic than determined by phage ELISA. Analysis in this format also revealed that the E2 mutations confer greatly enhanced affinity, and that the additional D4 and Met50Ile mutations (in construct HRG58) do not contribute additively. As observed in the phage ELISAs, the variants showed enhancements in affinity for ErbB-4-Ig that were similar to affinity-enhancements for ErbB-3-Ig. Variant affinities for ErbB-2/3-Ig were similar to the affinity of wild-type HRG- β 1 EGF for the 2/3-heterodimer, although the maximally substituted construct (HRG72) bound 6-fold more weakly to ErbB-2/3-Ig than did wild-type HRG- β 1 EGF.

The soluble variants were also assayed for their ability to stimulate tyrosine phosphorylation of the ErbB-2 receptor on MCF7 breast cancer carcinoma cells. This was accomplished in a KIRA-ELISA format as described in Sadick et al., *Analyt. Biochem.* 235:207-214, in which the ErbB-2 phosphorylation detected is

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(antiphosphotyrosine antibody) diluted to 0.2 μ g/ml in dilution buffer (PBS, 0.5% BSA, 0.05% Tween 20TM, 5 mM EDTA, 0.01% thimerosal) was added to each well. After incubation for 2 h at room temperature, the plate was washed, and 100 μ l horseradish peroxidase-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA), diluted 1:50000 in dilution buffer, was added to each well. The plate was incubated for 30 min at room temperature with gentle agitation. The free avidin conjugate was washed away, and 100 μ l freshly prepared substrate solution (tetramethyl benzidine, TMB, two-component substrate kit, Kirkegard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 min, after which the color development was stopped by the addition of 1.0 M H_3PO_4 .

The results are shown in Table 14. The EC_{50} is the concentration of variant (or HRG8) required to achieve 50% maximal tyrosine phosphorylation. In general, the EC_{50} 's for stimulation of phosphorylation by the variants tested did not differ substantially from the EC_{50} for wild-type HRG- β 1 EGF. In addition, the values correlated well with the IC_{50} 's for binding to the receptor fusions above.

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EXAMPLE 4

Selection of Heregulin- β 1 EGF Domain Variants

For ErbB-4 Receptor Binding

Using Monovalent Phage Display

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This example describes the selection of HRG- β 1 variants for binding to the ErbB-4 receptor, and in particular, variants having a greater specificity for the ErbB-4 receptor, relative to the ErbB-3 receptor, than wild-type HRG- β 1. Such variants have, for example, a lower variant:wild-type EC_{50} ratio for binding to ErbB-4-Ig than for binding to ErbB-3-Ig. The variants studied contained residues corresponding

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to HRG- β 1 177-244, which includes the minimal EGF-like domain (HRG- β 1 177-228). In this example, "HRG- β 1 EGF" refers to the region of the EGF-like domain extending from residues 177-244. Residue numbers are expressed in terms of position in 645-amino acid native human HRG- β 1 and, in parentheses, in terms of position in HRG- β 1 EGF (i.e., HRG- β 1 EGF 1-68).

The variants were produced by randomization at His178, Leu179, and Arg207 (His2, Leu3, and Arg31).

These residues were chosen because alanine scanning (Example 2) indicated that the substitution of alanine at these positions resulted in a significantly greater loss of affinity for the ErbB-3 receptor, as compared to the ErbB-4 receptor, suggesting that these residues may be more important for ErbB-3 receptor binding than for ErbB-4 receptor binding. Additionally, these residues are predicted to be proximal to one another on the surface of the HRG- β 1 molecule, potentially forming a binding site.

Phage Library Screening for ErbB-4-Ig Binding

Variants of HRG- β 1 EGF were prepared and selected for binding to ErbB-4-Ig using monovalent phage display, according to the method of Bass et al., *Proteins* 8:309-14 (1990). Briefly, the HRG- β 1 EGF phagemid vector was pHRG2-p3 (described in Example 1), in which HRG- β 1 177-244 was fused to a C-terminal fragment of the M13 coat protein pIII. Kunkel mutagenesis was performed to introduce stop codons into this vector at His178, Leu179, and Arg207 (His2, Leu3, and Arg31) to ensure that the starting vector could not express the wild-type polypeptide.

These sites were then randomized by Kunkel mutagenesis to produce an HRG- β 1 EGF library. Phage displaying mutated HRG- β 1 EGFs monovalently were produced from the library. See Bass et al., *supra*. These phage were then sorted against ErbB-4-Ig

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homodimers immobilized on an ELISA plate. Bound phage were eluted and used to reinfect host cells, which were used to produce new phage. Half of the phage from the first round of sorting was sorted against immobilized ErbB-4-Ig for three additional rounds of sorting. The other half of the phage from the first round was sorted against immobilized ErbB-4-Ig for three additional rounds in the presence of soluble ErbB-3-Ig (10 nM). Sorting the presence of soluble ErbB-3-Ig (i.e., "counter-selecting" against ErbB-3-Ig) was expected to remove variants with higher affinity for ErbB-3-Ig, allowing enrichment of those with higher affinity for ErbB-4-Ig in each round of sorting. After sorting, twelve clones from each of the two resultant libraries were sequenced.

Construction of Initial Phage Library

The initial phage library was constructed by Kunkel mutagenesis using uracil-containing single-stranded DNA template. TAA and TGA stop codons were installed at positions selected for randomization to generate a custom template that eliminated wild-type background from the pools. Positions were fully randomized by mutation to NNS codons (where N is any of the four bases and S is either G or C). Two mutagenesis oligonucleotides were used, one to randomize His178 and Leu179 (His2 and Leu3) and one to randomize Arg207 (Arg31). The oligonucleotides contained 15-base overhangs on either side of the randomized residues.

The final mutagenesis reaction mixture was electro-transformed into XL-1 blue cells (Stratagene, Inc., La Jolla, CA), according to the manufacturer's protocol. The transformed cells were then infected with 10^{11} pfu M13K07 helper phage (Promega Corp., Madison, WI), and phage stocks (about 10^{14} phagemid/mL) were prepared as described in Example 1.

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Table 15
Mutations and Affinities for ErbB-3- and ErbB-4-Igs
in Phage ELISA

5 Four Rounds of Sorting for ErbB-4-Ig Binding
(No Counter-Selection):

	Wild-type	H	L	R	#Siblings ^a	ErbB-4	ErbB-3
						EC ₅₀ Mut/WT ^b	EC ₅₀ Mut/WT
10	Clones	.	M	.	7	1.5	1.2
		W	.	.	2*	1.7	1.2
		E	.	.	2	1.7	9.5
		.	V	.	1	1.6	1.5

15 Four Rounds of Sorting for ErbB-4-Ig Binding
With ErbB-3-Ig Counter-Selection:

20	Clones	.	.	P	7	1.3	6.1
		L	.	.	1*	1.7	6.4
		L	.	.	1	1.2	1.4
		D	V	.	1	2.0	2.2
		E	V	.	1	1.7	0.8
25		T	M	.	1	1.6	1.4

*HRG- β 1 228-331 (HRG- β 1 EGF 52-55) were spontaneously deleted and replaced by a single Met residue.

30 ^aNumber of variants having this sequence (out of the twelve sequenced for each library).

35 ^bEC₅₀ for the variant divided by the EC₅₀ for wild-type HRG- β 1 177-244 expressed monovalently on phage

One variant predominated in each library, being represented in seven of the twelve clones sequenced from each library. The predominant variant from the library counter-selected against ErbB-3-Ig sorted back to the wild-type residue at positions His178 and Leu179 (His2 and Leu3) and had Pro at Arg207 (Arg31). The predominant variant from the library that was sorted without counter-selection sorted to wild-type at positions His178 and Arg207 (His2 and Arg31) and had Met at Leu179 (Leu3). In variants from each of the libraries, there was a spontaneous substitution of Met for HRG- β 1 228-231 (HRG- β 1 EGF 52-55).

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Analysis of the Impact of Mutations on Receptor Affinity and Specificity

Receptor binding affinities were measured by phage ELISA for all unique variants from each library, as described in Example 1. The results are shown in Table 15. The affinities of the variants for ErbB-3-Ig and ErbB-4-Ig were determined to assess specificity.

The affinities of most variants for the ErbB receptor-Ig fusions were reduced between about 1.2-fold and 2.2-fold. Only one variant showed a slight enhancement in affinity, and only for ErbB-3-Ig. However, three variants had affinities for ErbB-4-Ig that were less than two-fold below the affinity of wild-type HRG- β 1, whereas their affinities for ErbB-3-Ig were 6- to 9-fold below the affinity of wild-type HRG- β 1. All of these variants sorted back to the wild-type residue at Leu179 (Leu3). One variant sorted to Glu at His178 (His2) and to wild-type at Arg207 (Arg31). Another variant sorted to wild-type at His178 (His2) and to Pro at Arg 207 (Arg31). This was the predominant variant in the counter-selected library.

The third variant sorted to Leu at His178 (His2) and to wild-type at Arg207 (Arg31) and also had the spontaneous substitution of Met for HRG- β 1 228-231 (HRG- β 1 EGF 52-55). The affinity of this variant for ErbB-3-Ig was reduced 6.4 fold, whereas another variant with the same sequence at His178, Leu179, and Arg207 (His2, Leu3, and Arg31), but lacking the spontaneous substitution, retained approximately wild-type affinity for ErbB-3-Ig. Thus, the reduction in affinity for ErbB-3-Ig binding is largely attributable to this spontaneous substitution. Because HRG- β 1 228-231 (HRG- β 1 EGF 52-55) is unlikely to be proximal to the randomized region on the surface of the HRG- β 1 molecule, the data suggest that at least two distinct regions of HRG- β 1 are important for ErbB receptor

Two of the three variants exhibiting enhanced specificity for ErbB-4-Ig binding accounted for eight of the twelve clones isolated from the counter-selected library. The other variant accounted for only two of the twelve clones isolated from the library that was sorted without counter-selection. Thus, counter-selection against ErbB-3-Ig produced a significant enrichment in variants exhibiting greater specificity for ErbB-4-Ig, relative to ErbB-3-Ig, than that of wild-type HRG- β 1.

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SEQUENCE LISTING

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Fairbrother, Wayne J.
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10 Wells, James A.
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- (ii) TITLE OF INVENTION: HEREGULIN VARIANTS
- 15 (iii) NUMBER OF SEQUENCES: 92
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(C) CITY: San Jose
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(F) ZIP: 95110
- 25 (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
30 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Pending
(B) FILING DATE: 10-FEB-1998
- 35 (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/799,054
(B) FILING DATE: 10-FEB-1997
(C) CLASSIFICATION:
- 40 (viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 38,903
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- 45 (ix) TELECOMMUNICATION INFORMATION:
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- 50 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid

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(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
20 Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
35 40 45
Val Met Ala Ser Phe Tyr Lys His Leu Gly Ile Glu Phe Met Glu Ala
50 55 60
25 Glu Glu Leu Tyr Gln Lys Arg
65 70

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

45 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
50 Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
35 40 45

Val Pro Met Lys Val Gln Asn Gln Glu Lys Ala Glu Glu Leu Tyr Gln
 50 55 60

5 Lys Arg
 65

(2) INFORMATION FOR SEQ ID NO:3:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 63 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

15 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

- 20 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

25 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
 1 5 10 15
 30 Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
 20 25 30
 Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
 35 40 45
 35 Val Met Ala Ser Phe Tyr Lys Ala Glu Glu Leu Tyr Gln Lys Arg
 50 55 60

(2) INFORMATION FOR SEQ ID NO:4:

- 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 65 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

45 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

- 50 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

403426 v1

Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
5 Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
35 40 45
10 Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro
50 55 60
Glu
15 65

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
20 (A) LENGTH: 66 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

25 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
30 (A) ORGANISM: Rattus rattus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

35 Ser His Leu Ile Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Thr Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
40 Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
35 40 45
Val Pro Met Lys Val Gln Thr Gln Glu Lys Ala Glu Glu Leu Tyr Gln
45 50 55 60
Lys Arg
65

50 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid

$$\frac{1}{\Gamma(\alpha)} \int_0^t (t-\tau)^{\alpha-1} f(\tau) d\tau = \frac{1}{\Gamma(\alpha)} \int_0^t (t-\tau)^{\alpha-1} \left(\sum_{k=0}^{\infty} \frac{f^{(k)}(0)}{k!} \tau^k \right) d\tau$$

(D) TOPOLOGY: not relevant

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(vi) ORIGINAL SOURCE:

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Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
35 40 45

403426 v1

Val Met Ala Ser Phe Tyr Lys Ala Glu Glu Leu Tyr Gln Lys Arg
50 55 60

5 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 amino acids
(B) TYPE: amino acid
10 (C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

15 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus rattus

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser His Leu Ile Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
25 Gly Gly Glu Cys Phe Thr Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
30 Leu Cys Lys Cys Gln Pro Gly Phe Thr Gly Ala Arg Cys Thr Glu Asn
35 40 45
Val Pro Met Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro Glu
50 55 60

35

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 81 amino acids
40 (B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

45

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus rattus

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser His Leu Ile Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn

(2) INFORMATION FOR SEQ ID NO:10:

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

(2) INFORMATION FOR SEQ ID NO:11:

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(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

5 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

15 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
35 40 45
Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro
25 50 55 60
Glu
65

30 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

35 (ii) MOLECULE TYPE: protein

40 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

50 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr

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35 40 45

Val Met Ala Ser Phe Tyr Ser Thr Ser Thr Pro Phe Leu Ser Leu Pro
50 55 60

5
Glu
65

(2) INFORMATION FOR SEQ ID NO:13:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
15 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

20 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Gallus domesticus

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser His Leu Thr Lys Cys Asp Ile Lys Gln Lys Ala Phe Cys Val Asn
1 5 10 15

30 Gly Gly Glu Cys Tyr Met Val Lys Asp Leu Pro Asn Pro Pro Arg Tyr
20 25 30

Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
35 40 45

Val Met Ala Ser Phe Tyr Lys His Leu Gly Ile Glu Phe Met Glu Ala
50 55 60

Glu Glu Leu Tyr Gln Lys Arg
40 65 70

(2) INFORMATION FOR SEQ ID NO:14:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

50 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn
1				5					10					15	
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Pro	Ser	Arg	Tyr	Leu	Cys	Lys
			20					25					30		
Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala
		35					40					45			

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

25 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

30 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

```

35      Asn Ser Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr Cys Leu His
      1              5              10              15

      Asp Gly Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn
40              20              25              30

      Cys Val Val Gly Tyr Ile Gly Glu Arg Cys Gln Tyr Arg Asp Leu Arg
              35              40              45

```

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

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(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

5 (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

10 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Met Val Lys Asp Pro Ser Arg Tyr Leu Cys Lys
20 25 30
15 Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
35 40 45
20 Ser

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 52 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

30 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

35 (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

40 Trp Glu Leu Val Pro Cys Gly Trp Asp Arg Glu Gly Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
45 Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
35 40 45
50 Val Ile Ala Ser
50

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

5

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

10

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

15

Trp Glu Leu Val Pro Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15

20

Gly Gly Glu Cys Tyr Lys Val Arg Ile Tyr Gly Tyr Leu Met Cys Lys
20 25 30

Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
35 40 45

25

Ser

(2) INFORMATION FOR SEQ ID NO:19:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

35

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

40.

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

45

Trp Glu Leu Val Pro Cys Gly Trp Asp Arg Glu Gly Phe Cys Val Asn
1 5 10 15

50

Gly Gly Glu Cys Tyr Lys Val Arg Ile Tyr Gly Tyr Leu Met Cys Lys
20 25 30

Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
35 40 45

(2) INFORMATION FOR SEQ ID NO:20:

5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

10

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

15

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Trp Glu Leu Val Pro Cys Gly Trp Asp Arg Glu Gly Phe Cys Val Asn
1 5 10 15

25

Gly Gly Glu Cys Tyr Lys Val Arg Ile Tyr Arg Tyr Arg Met Cys Lys
20 25 30

Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
35 40 45

30

Ser

(2) INFORMATION FOR SEQ ID NO:21:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

40

(ii) MOLECULE TYPE: peptide

(iii) ~~HYPOTHETICAL~~: NO

45

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1. 5 10 15

Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
35 40 45

10 (2) INFORMATION FOR SEQ ID NO:22:

15

20 (iii) HYPOTHETICAL: NO

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

30

35

Val Met Ala Ser
50

40 (2) INFORMATION FOR SEQ ID NO:23:

45

50 (iii) HYPOTHETICAL. NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

5 Ser His Leu Val Lys Cys Gly Glu Glu Arg Glu Gly Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Met Val Lys Asp Tyr Gly Tyr Leu Met Cys Lys
20 25 30
10 Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala
35 40 45
Ser

15

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
20 (A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant
(ii) MOLECULE TYPE: peptide
25 (iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

35 Ser His Leu Val Lys Cys Gly Glu Glu Arg Glu Gly Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Tyr Arg Val Lys Thr Tyr Gly Tyr Leu Met Cys Lys
20 25 30
40 Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala
35 40 45
Ser

45

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
50 (A) LENGTH: 52 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

403426 v1

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

5 (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

10 Ser His Leu Val Lys Cys Gly Glu Glu Arg Glu Gly Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr
20 25 30
15 Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
35 40 45
Val Ile Ala Ser
20 50

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

30 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

35 (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

40 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15
Gly Gly Glu Cys Tyr Arg Val Lys Thr Tyr Gly Tyr Leu Met Cys Lys
20 25 30
45 Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Met Ala
35 40 45
Ser
50

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

403426 v1

- (A) LENGTH: 49 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

5

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

10

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15

20

Gly Gly Glu Cys Tyr Arg Val Lys Thr Tyr Gly Tyr Leu Met Cys Lys
20 25 30

Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
35 40 45

25

Ser

(2) INFORMATION FOR SEQ ID NO:28:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

35

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

40

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15

50

Gly Gly Glu Cys Tyr Arg Val Lys Thr Leu Ser Asn Pro Ser Arg Tyr
20 25 30

Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr

35 40 45
Val Ile Ala Ser
50

5

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 52 amino acids
 10 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: peptide
 15
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Not relevant (recombinant)
 20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

25 Ser His Leu Val Lys Cys Gly Glu Glu Arg Glu Gly Phe Cys Val Asn
 1 5 10 15
 Gly Gly Glu Cys Tyr Arg Val Lys Thr Leu Ser Asn Pro Ser Arg Tyr
 20 25 30
 Leu Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr
 35 40 45
 Val Ile Ala Ser
 35 50

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 40 (A) LENGTH: 49 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: peptide
 45
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 50 (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

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Ser His Leu Val Lys Cys Gly Glu Glu Arg Glu Gly Phe Cys Val Asn
1 5 10 15
5 Gly Gly Glu Cys Phe Met Val Lys Asp Tyr Gly Tyr Leu Met Cys Lys
20 25 30
Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
35 40 45
10 Ser

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ser His Leu Val Lys Cys Gly Glu Glu Arg Glu Gly Phe Cys Val Asn
1 5 10 15
35 Gly Gly Glu Cys Tyr Arg Val Lys Thr Tyr Gly Tyr Leu Met Cys Lys
20 25 30
Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln Asn Tyr Val Ile Ala
40 35 40 45
Ser

(2) INFORMATION FOR SEQ ID NO:32.

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

5

10 Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
1 5 10 15

Gly Gly Glu Cys Tyr Arg Val Lys Thr Tyr Gly Tyr Leu Met Cys Lys
20 25 30

15

Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln His Tyr Val Ile Ala
35 40 45

Ser

20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 49 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

30

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

35

(A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

40

Ser His Leu Val Lys Cys Gly Glu Glu Arg Glu Gly Phe Cys Val Asn
1 5 10 15

Gly Gly Glu Cys Tyr Arg Val Lys Thr Tyr Gly Tyr Leu Met Cys Lys
20 25 30

45

Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln His Tyr Val Ile Ala
35 40 45

50

Ser

(2) INFORMATION FOR SEQ ID NO:34:

5

(iii) HYPOTHETICAL: NO

10

15

20

25

30

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45

50

(iii) HYPOTHETICAL: NO

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(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Thr Arg Asp Lys Thr
1 5

10

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

15

(ii) MOLECULE TYPE: peptide

20

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

30

Asp Asp Asp Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO:38:

35

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

40

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

45

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Ser His Leu Val Lys
1 5

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(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Not relevant (recombinant)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Trp	Arg	Leu	Val	Pro
1				5

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Not relevant (recombinant)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Trp	Ser	Leu	Gln	Pro
1				5

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: peptide

5

(A) ORGANISM: Not relevant (recombinant)

10

(2) INFORMATION FOR SEQ ID NO:42:

20

(iii) HYPOTHETICAL: 'NO

30

35

(2) INFORMATION FOR SEQ ID NO:43:

40

45

(iii) HYPOTHETICAL: NO.

(A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Trp Ser Leu Ile Pro

5

(2) INFORMATION FOR SEQ ID NO:44:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

10 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

15 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Trp Arg Leu Val Ala
1 5

25 (2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids .
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

35 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

45 Trp-Ala Leu Val Pro
1 5

(2) INFORMATION FOR SEQ ID NO:46:

50 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

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- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- 5 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:
Trp Ser Leu Gln Lys
1 5
- 15 (2) INFORMATION FOR SEQ ID NO:47:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
20 (C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: peptide
- 25 (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)
- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:
Trp Glu Leu Val Ala
35 1 5
- (2) INFORMATION FOR SEQ ID NO:48:
- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant
- 45 (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
50 (A) ORGANISM: Not relevant (recombinant)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

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Trp Ser Leu Glu Pro
1 5

5 (2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 10 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

15 (iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Ala Glu Lys Glu Lys Thr
1 5

25

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 30 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

35 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Not relevant (recombinant)

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Gly Val Gly Arg Asp Gly
1 5

45

(2) INFORMATION FOR SEQ ID NO:51:

50

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant

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(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

5 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Gly Gly Glu Arg Glu Gly

15

1 5

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

20

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

25

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

30

(A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

35

Gly Glu Glu Arg Glu Gly

1 5

(2) INFORMATION FOR SEQ ID NO:53:

40

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

45

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

50

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

5 Gly Trp Asp Arg Glu Gly
1 5

(1) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(vi) ORIGINAL SOURCE:
20 (A) ORGANISM: Not relevant (recombinant)

Gly Val Gln Arg Glu Gly
1 5

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

Gly Glu Glu Arg Ala Gly
1 5

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids

5 (ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
10 (A) ORGANISM: Not relevant (recombinant)

(2) INFORMATION FOR SEQ ID NO:57:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

25 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

30 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

50 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Val Asn Gly Gly Val
1 5

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(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

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(ii) MOLECULE TYPE: peptide

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(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Val Asn Gly Gly Gln
1 5

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(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

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(ii) MOLECULE TYPE: peptide

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(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Phe Met Val Lys Asp
1 5

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(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Tyr Lys Val Arg Ile
1 5

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Phe Arg Val Lys Thr
1 5

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

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10      Tyr Arg Val Lys Thr
      1              5

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(2) INFORMATION FOR SEQ ID NO:69:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

25 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant).

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Tyr Met Ile Lys Tyr
1 5

35 (2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

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(ii) MOLECULE TYPE: peptide

45 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Tyr Met Val Lys Thr

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1 5

(2) INFORMATION FOR SEQ ID NO:71:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant
- 10 (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- 15 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Not relevant (recombinant)

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Met Arg Val Arg Thr
 1 5'

25 (2) INFORMATION FOR SEQ ID NO:72:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant
- 30 (ii) MOLECULE TYPE: peptide
- 35 (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

Pro Ser Arg Tyr Leu
 1 5

(2) INFORMATION FOR SEQ ID NO:73:

- 50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

(iii) HYPOTHETICAL: NO

5 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

Thr Pro Tyr Leu Met
1 5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

25 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

35 Tyr Gly Tyr Leu Met
1 5

40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
50 (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

5 (2) INFORMATION FOR SEQ ID NO:76:

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15 (iii) HYPOTHETICAL: NO

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Thr His Tyr Arg Gly .
25 1 5

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(iii) HYPOTHETICAL: NO

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(2) INFORMATION FOR SEQ ID NO:78:

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(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

5 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

Tyr Lys Tyr Arg Met

15 1 5

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: not relevant

25 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

30 (A) ORGANISM: Not relevant (recombinant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

35 Thr Lys Tyr Arg Gly

1 5

(2) INFORMATION FOR SEQ ID NO:80:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

45 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

50 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

5 Tyr Lys Tyr Arg Leu
1 5

(2) INFORMATION FOR SEQ ID NO:81:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

15 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

20 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

25 Lys Cys Pro Asn Glu Phe
1 5

(2) INFORMATION FOR SEQ ID NO:82:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
35 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

40 (vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

Arg Cys Ser Leu Glu Phe
1 5

50 (2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids

5 (ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(vi) ORIGINAL SOURCE:
10 (A) ORGANISM: Not relevant (recombinant)

15 Arg Cys Ser Glu Glu Phe
1 5

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 25 (D) TOPOLOGY: not relevant

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

Lys Cys Pro Lys Glu Met
1 5

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

50 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Arg Cys Thr Val Glu Tyr
1 5

10 (2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
15 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

20 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Arg Cys Thr Val Glu Tyr
1 5

30 (2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
35 (B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

40 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Not relevant (recombinant)

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

50 Lys Cys Asn Ser Glu Phe
1 5

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
5 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

10 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Not relevant (recombinant)

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

 Arg Cys Lys Lys Glu Phe
 1 5

20 (2) INFORMATION FOR SEQ ID NO:89:

 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
25 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
 (D) TOPOLOGY: not relevant

 (ii) MOLECULE TYPE: peptide

30 (iii) HYPOTHETICAL: NO

 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

40 Gln Asn Tyr Val Met
 1 5

 (2) INFORMATION FOR SEQ ID NO:90:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: not relevant
50 (D) TOPOLOGY: not relevant

 (ii) MOLECULE TYPE: peptide

 (iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

Gln Trp Tyr Val Ile
 1 5

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(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPL: peptide

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(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Not relevant (recombinant)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

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Gln His Tyr Val Ile
 1 5

(2) INFORMATION FOR SEQ ID NO:92:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 52 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Ser His Leu Val Lys Cys Ala Glu Lys Glu Lys Thr Phe Cys Val Asn
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	Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr
				20					25					30		
5	Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr
		35						40					45			
	Val	Met	Ala	Ser												
		50														